The role of Netrin-1 in semicircular canal morphogenesis

The role of Netrin-1 in semicircular canal morphogenesis

A dissertation presented

by

Allison Nishitani

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Neurobiology

Harvard University

Cambridge, Massachusetts

July 2014

Dissertation Advisor: Dr. Lisa Goodrich **Allison Nishitani**

The role of Netrin-1 in semicircular canal morphogenesis

Abstract

The vestibular system of the inner ear detects head position using three orthogonally oriented semicircular canals. Vestibular function relies on precise canal shape and orientation, and slight changes can cause vestibular defects. Canals are sculpted from pouches that protrude from the otic vesicle, the simple sphere of epithelium that forms the inner ear. In the center of each pouch, a “fusion plate” forms where cells lose their epithelial morphology and the basement membrane breaks down. The opposing layers of the fusion plate intercalate and are subsequently removed, creating a canal. Proper fusion depends on Netrin-1, which regulates basement membrane breakdown during fusion in mice, although the underlying molecular mechanism is unknown. This dissertation describes our work to better understand the cellular effects of Netrin-1 during canal formation.

Although vestibular apparatus structure is shared among species, some developmental events that lead to this structure differ. For example, while fusion plate basement membrane breakdown is conserved, apoptosis is required for fusion in chicks, but not in mice. We used gain-of-function approaches to determine the main cellular effect of Netrin-1 during fusion in chicks and mice. We show that overexpression of Netrin-1 in chicks prevents canal fusion from occurring normally by interfering with apoptosis. On the other hand, we show that ectopic expression of Netrin-1 in mice using a conditional expression allele causes excessive fusion, resulting in canal truncation.

This suggests that Netrin-1 may play divergent roles during canal morphogenesis in chicks and mice.

To determine if Netrin-1 regulates the basement membrane in other contexts, we created a *Netrin-1* conditional null allele. This was necessary because existing *Netrin-1* mutants express residual Netrin-1 protein, which could be sufficient to rescue basement membrane defects in other tissues, and because existing mutants die shortly after birth, preventing postnatal analysis. Complete loss of Netrin-1 protein in our newly generated mice does not cause more severe defects in fusion compared to existing *Netrin-1* hypomorphs, suggesting that residual Netrin-1 protein does not affect the basement membrane during fusion in *Netrin-1* hypomorphs. Future work will determine if complete loss of Netrin-1 affects basement membrane integrity in other tissues.

Table of contents

Acknowledgements

I would like to thank my advisor, Dr. Lisa Goodrich, for her support and mentorship throughout my graduate career. Her boundless enthusiasm for science and positive attitude buoyed me throughout graduate school. I would also like to thank all the members of the Goodrich lab for teaching me numerous scientific techniques and for their intellectual contributions and critical analysis of my work. In particular, I must acknowledge my baymate, Wei-Ming Yu, for immeasurable technical support, as well as emotional support in the form of silly animal videos. I am also extremely grateful to my classmate and labmate, Sasha Krol, for her scientific and personal comradery. Finally, I must thank Andrea Yung for joining me in the study of Netrin-1 at the end of my graduate career.

I am grateful for administrative support from the Program in Neuroscience, the Division of Medical Sciences, and the Department of Neurobiology. I am also very thankful to Drs. Zhigang He, Roz Segal, and Davie Van Vactor for serving on my Preliminary Qualifying Examination Committee and Dissertation Advisory Committee. Their scientific and personal advice throughout the years helped guide both my research and future plans. I particularly thank Dr. Roz Segal for serving as the chair of my Dissertation Examination Committee, as well as Drs. Corey Harwell, Sean Megason, and Doris Wu for their participation.

Finally, thanks to my family and friends for their support. In particular, I must thank Dan Berman for keeping me grounded and for proofreading this dissertation. This work was supported by a Harvard Medical School Edward R. and Anne G. Lefler Fellowship.

Chapter 1: General introduction

Inner ear function

The inner ear is a membranous, fluid-filled labyrinth encased in bone that is divided into two functional systems: the cochlea is responsible for hearing and the vestibular apparatus is responsible for balance. Although they mediate different senses, both systems rely on mechanosensitive hair cells to transduce changes in fluid movement caused by sound waves or head movement into neural signals. Fluid vibrations in the cochlea caused by sound waves are transduced into neural signals by mechanosensitive hair cells in the organ of Corti. Similarly, the vestibular apparatus detects acceleration using mechanosensitive hair cells located in distinct sensory epithelia of its five main regions: the three fluid-filled semicircular canals, the utricle, and the saccule. The semicircular canals are oriented in the three orthogonal planes and contain a swelling at their base called an ampulla. Within each ampulla is a sensory epithelium known as the crista, which contains mechanosensitive hair cells that respond to fluid flow through the canal caused by angular acceleration of the head. Similarly, the utricle and saccule detect linear acceleration using mechanosensitive hair cells located in sensory epithelia known as maculae (Figure 1.1).

While hair cells transduce physical stimuli into neural signals, specialized relay neurons send this information to the auditory brainstem. The spiral ganglion coils along the cochlea and innervates auditory hair cells, while the vestibular ganglion innervates vestibular hair cells. Spiral and vestibular ganglion neurons must faithfully transmit all auditory and vestibular information from the periphery to the brain for perception and higher order processing.

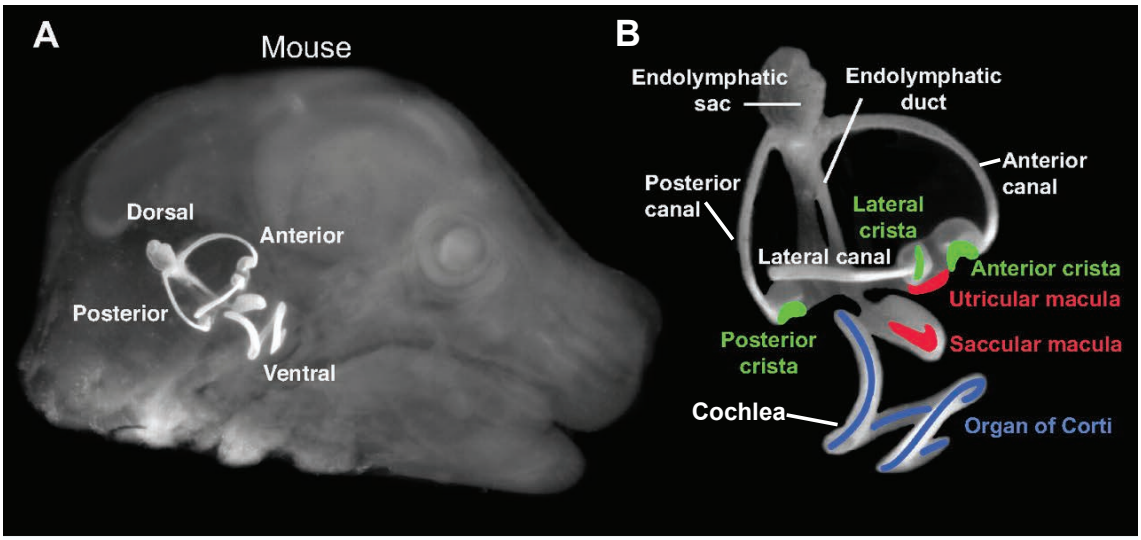


Figure 1.1: Anatomy of the mouse inner ear. A) A paintfill of the mouse inner ear shows the positon of the organ within the head. B) The locations of the sensory epithelia within the inner ear are illustrated. For the vestibular system, the crista of the semicircular canals are shown in green and the macula of the utricle and saccule are shown in red. For the auditory system, the organ of Corti is shown in blue. Figure modified from Groves and Fekete, 2012. Reprinted with permission.

Inner ear development

In mice, inner ear development begins between E8 and E8.5 with the appearance of an ectodermal thickening adjacent to the hindbrain called the otic placode (Figure 1.2 A). Wnts and Fgfs from surrounding tissues induce the otic placode, but the specific signaling molecules used and the tissues that secrete them vary among species (reviewed in Wu and Kelley, 2012; Groves and Fekete 2012). At E9, the otic placode invaginates to form the otic cup (Figure 1.2 B), which deepens and detaches to form the otic vesicle by E9.5 (Figure 1.2 C) (Anniko and Wikstrom, 1984). The otic vesicle begins as a simple sphere of epithelium separated from the surrounding mesenchyme by a basement membrane. Signals from the hindbrain and surrounding mesenchyme pattern the otic vesicle along both the anterior/posterior and dorsal/ventral axes (reviewed in Bok and Wu, 2007). In amniotes, retinoic acid patterns the anterior/posterior axis: low retinoic acid anteriorly leads to a sensory fate and high retinoic acid posteriorly leads to a non-sensory fate (reviewed in Wu and Kelley, 2012; Groves and Fekete, 2012). Wnt and Shh pattern the dorsal/ventral axis: Wnts from the dorsal hindbrain promote a vestibular fate while Shh from the ventral floorplate and notochord promote an auditory fate (reviewed in Wu and Kelley, 2012; Groves and Fekete, 2012). After this basic patterning, precisely coordinated changes in cell proliferation, apoptosis, and epithelial to mesenchymal transitions sculpt the otic vesicle into the complex structure of the mature inner ear (Figure 1.3).

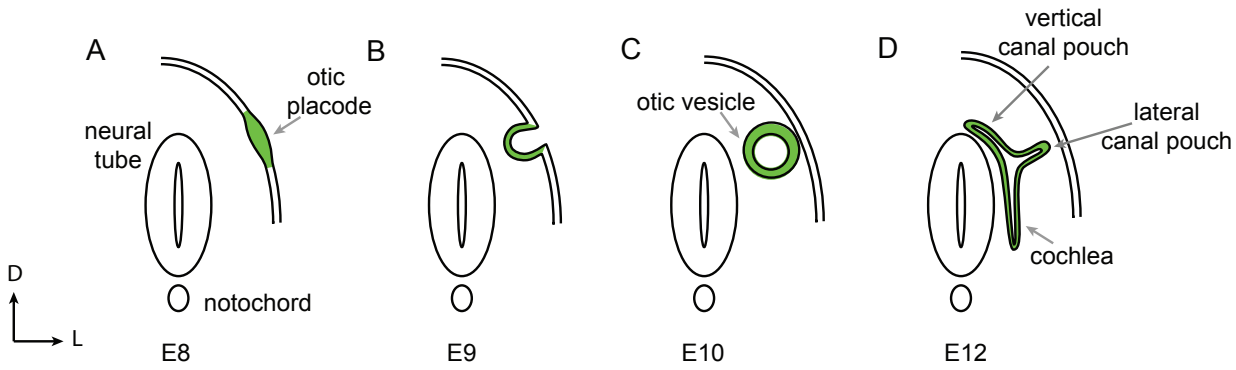


Figure 1.2 Early inner ear development. The otic placode (A) is an ectodermal thickening, which invaginates (B) to form the otic vesicle (C). Pouches grow out from the dorsal otic vesicle (D), which will give rise to the semicircular canals.

Semicircular canal morphogenesis

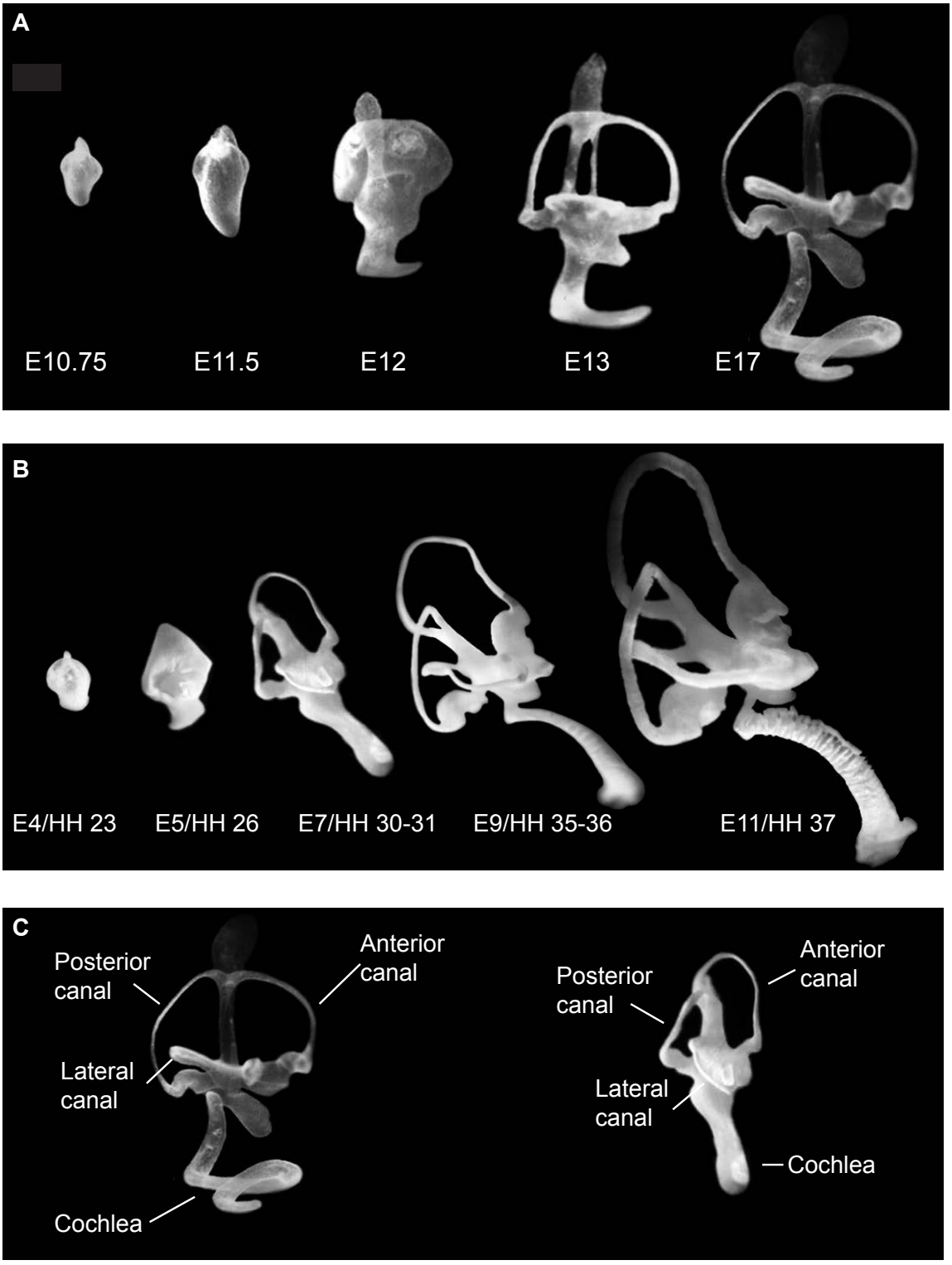
The work presented in this dissertation focuses on the development of the vestibular apparatus, specifically the formation of the semicircular canals. The precise orientation of the three semicircular canals (anterior, posterior, and lateral) underlies the inner ear's ability to perceive the position of the head in space. Even slight changes in canal structure lead to severe vestibular defects such as vertigo, dizziness, and abnormal posture (Sando et al., 2001).

Canal morphogenesis in amniote vertebrates begins when epithelial pouches begin to protrude from the fairly round otic vesicle. A vertical canal pouch forms the anterior and posterior canals, while a separate lateral canal pouch forms the lateral canal (Figure 1.2 D). These simple epithelial pouches transform into the intricately shaped semicircular canals through a process called fusion. In the center of each pouch, special areas called fusion plates form where the opposing epithelial walls of the canals meet and fuse into a single layer. These cells are then cleared away leaving a donut shaped canal (Figure 1.4). Fusion in anamniote fish and frogs proceeds differently: epithelial protrusions grow out from the walls of the otic vesicle, meet, and fuse to form epithelial pillars, which separate the otic vesicle into the three semicircular canals (Waterman and Bell, 1984; Paterson,1948). Although the geometry varies, the fusion of epithelial layers during canal morphogenesis is conserved across vertebrates.

The cellular events that accompany fusion have been described (Figure 1.4), but they remain poorly understood at the molecular level. When fusion initiates, the cells at the fusion plate lose their epithelial morphology and the underlying basement membrane breaks down (Martin and Swanson, 1993). Proliferation in the surrounding

Figure 1.3: Inner ear morphogenesis in mice and chicks. Paintfilling the inner ear at various developmental stages demonstrates gross inner ear morphogenesis. A) During mouse development, the inner ear transforms from the simple otic vesicle (shown at E10.75) to its complex mature form (shown at E17). The vestibular system develops from the dorsal portion of the otic vesicle, while the auditory cochlea grows out ventrally. B) Although the final structure varies somewhat, chick inner ear morphogenesis proceeds similarly to the mouse. The inner ear transforms from the simple otic vesicle (shown at E4) to its complex mature form (shown at E11). The vestibular system develops from the dorsal portion of the otic vesicle, while the auditory cochlea grows out ventrally. C) While the exact form of the inner ear differs between mice and chicks, each contains vestibular three semicircular canals and the auditory cochlea. Figure reproduced with permission from Morsli et al, 1998 and images provided by D. Fekete.

Figure 1.3 (Continued)



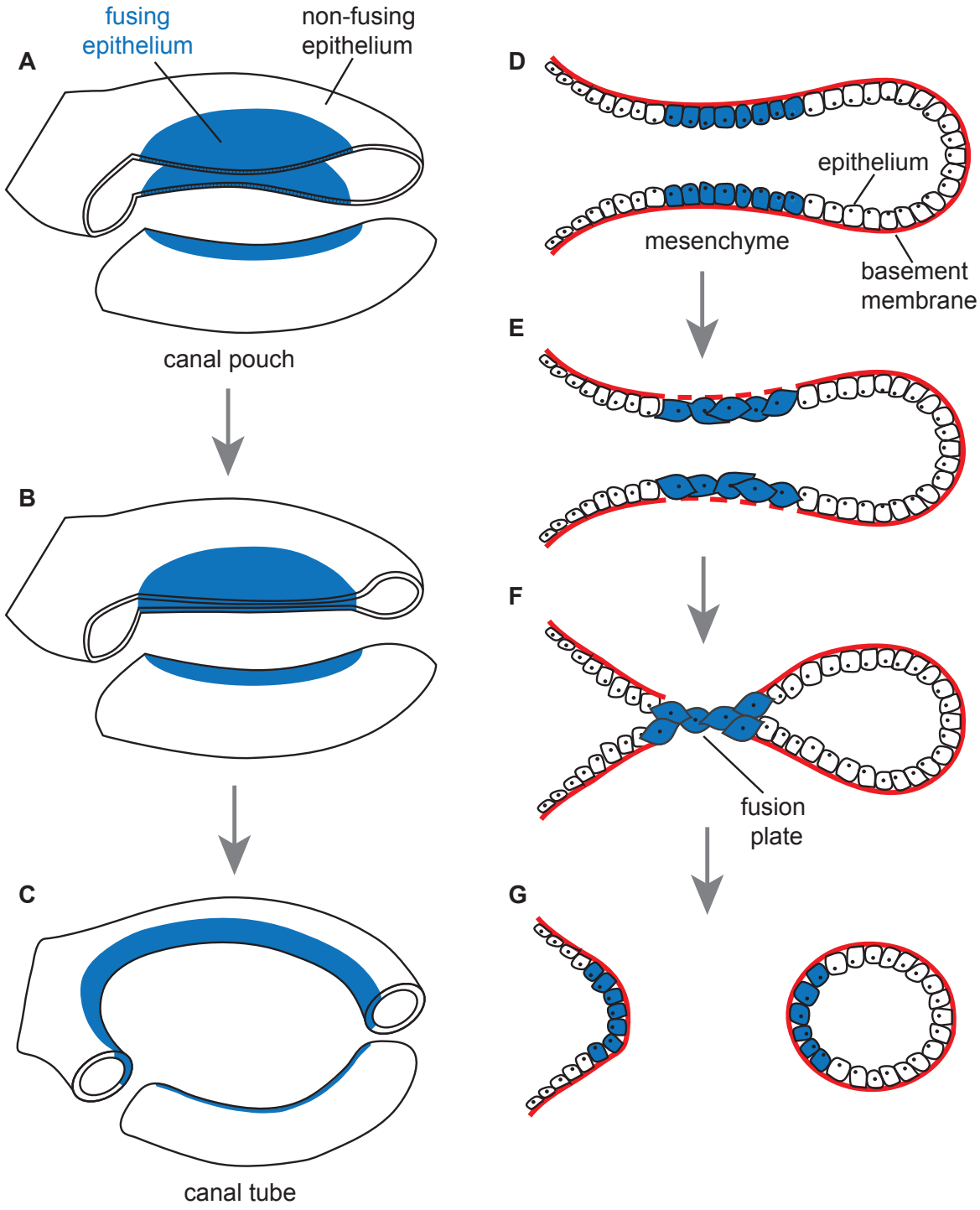
mesenchyme is proposed to push the opposing epithelial layers together (Pirvola et al., 2004), enabling them to subsequently intercalate into a single layer. Finally, the fusion plate cells are cleared away and mesenchyme fills the space.

Three mechanisms are proposed for fusion plate cell clearance: recruitment of the fusion plate cells back into the rim of the canal epithelium, epithelial to mesenchymal transition, and apoptosis. While fate-mapping studies will be necessary to definitively determine the fate of fusion plate cells, a small amount of observational information has been collected on this process. Martin and Swanson used dye labeling to mark canal pouch epithelial cells before fusion and looked at tissue sections just after fusion was complete. No dye-labeled mesenchymal cells were observed adjacent to the former fusion plate, suggesting that epithelial-to-mesenchymal transition does not occur during canal fusion. Moreover, no phagocytes containing dye-labeled cell debris were seen, suggesting that apoptosis is not a primary mechanism for clearing fusion plate cells. The lack of evidence for epithelial-to-mesenchymal transition and apoptosis led the authors to conclude that fusion plate cells were recruited back into the canal epithelium (Martin and Swanson, 1993). While no other evidence exists concerning epithelial-to-mesenchymal transition or epithelial recruitment during canal fusion in mice, other studies have supported the absence of appreciable levels of cell death at the fusion plate in mice (Nishikori et al., 1999).

Unlike in the mouse, however, apoptosis is required for fusion in the chick: many apoptotic cells are present at the fusion plate, and blocking cell death by overexpressing Bcl2 interferes with fusion plate clearing, resulting in wider diameter canals, or the retention of a canal pouch (Fekete et al., 1997). A histological analysis of fusion in the

Figure 1.4: Semicircular canals form through a fusion process. A) At the gross level, semicircular canal formation begins when epithelial outgrowths called canal pouches protrude from the otic vesicle. The center of the canal pouch is composed of epithelium that will undergo fusion (blue) while the edge of the canal pouch is composed of epithelium that will remain unfused (white). B) In the center of the canal pouch, the opposing epithelial layers come together and fuse into a single layer, forming a fusion plate. C) The fusion plate is then cleared away to create the donut shape of the mature canal. D) At the cellular level, the canal pouch is composed of the otic epithelium, which is separated from the surrounding mesenchyme by the basement membrane (red). E) As fusion begins, the cells in the center of the canal pouch lose their epithelial morphology and the underlying basement membrane breaks down. F) The opposing epithelial layers then come together to form the fusion plate. G) The fusion plate cells are removed, and the epithelial cells form the intact epithelium of the tubular canals.

Figure 1.4 (Continued)



chick revealed cells with a mesenchymal morphology adjacent to the degraded basement membrane at the former fusion plate, suggesting that some fusion plate cells transition to mesenchymal cells (Kobayashi et al., 2008). Further, the basement membrane was observed to reform around the newly formed canal leaving many fusion plate cells outside. If recruitment into the canal epithelium were the major fate of fusion plate cells then these cells would have to traverse the basement membrane to incorporate into the canal epithelium (Kobayashi et al., 2008).

One of the few molecules known to be involved in fusion is the axon guidance molecule, Netrin-1. In the mouse*, Netrin-1* is expressed in the region of the otic epithelium that will form the fusion plate beginning at E10.5 (Salminen et al., 2000; Matilainen et al., 2007). *Netrin-1* expression continues at the fusion plate throughout canal formation and it remains expressed in the canal rim postnatally (Salminen et al., 2000). Netrin-1 is required for canal formation in mice: in *Netrin-1* mutants, the basement membrane at the fusion plate remains intact, proliferation in the surrounding mesenchyme is reduced, and canal formation is arrested before fusion occurs (Salminen et al., 2000). On the other hand, in mice mutant for *Lrig3*, a protein that restricts *Netrin-1* expression to the fusion plate in the lateral canal pouch, *Netrin-1* is ectopically expressed throughout the lateral canal pouch epithelium. In these mice, the expanded *Netrin-1* expression causes early and expanded basement membrane breakdown and fusion, resulting in a lateral canal truncation. Removing one copy of *Netrin-1* rescues the lateral canal truncation in *Lrig3* mutants, suggesting that the *Lrig3* phenotype is caused by ectopic *Netrin-1* (Abraira et al., 2008). Together, these

observations demonstrate that Netrin-1 regulates basement membrane breakdown during canal fusion although the underlying mechanism remains a mystery.

Netrin protein family

Netrin proteins (Figure 1.5) are roughly 600 amino acids in size and have structural similarities to the extracellular matrix protein laminin (Yurchenco and Wadsmorth, 2004). The N-termini of Netrins resemble laminin domains VI and V (Serafini et al., 1994), while the makeup of the C-termini varies. Secreted Netrins contain a C-terminal Netrin-like (NTR) domain that is not related to laminin, but shares homology with tissue inhibitors of metalloproteinases (TIMPs), complement C3, 4, and 5, secreted frizzled related proteins (sFRPs), and type I C-proteinase enhancer proteins (PCOLCEs) (Banyai and Patthy, 1999). The NTR domain has repeated basic residues (Kappler et al., 2000) and binds extracellular matrix components such as heparin sufate proteoglycans (Geisbrecht et al., 2003; Kappler et al., 2000), laminin (Schneiders et al., 2007), and type IV collagen (Yebra et al., 2003).

The first Netrin family member, Unc6, was discovered in worms due to a defect in circumferential axon guidance (Hedgcock et al., 1990; Ishii et al., 1992). All bilaterally symmetric animals studied so far express an isoform of Netrin-1 at the midline, including the sea anemone *Nematostella vactensis* (Matus et al., 2006), the fly *Drosophila melanogaster* (Harris et al., 1996; Mitchell et al., 1996), the frog *Xenopus laevis* (de la Torre 1997), the fish *Danio rerio* (Lauderdale et al., 1997; Strahle et al., 1997), the chicken *Gallus gallus* (Serafini et al., 1994), the mouse *Mus musculus* (Serafini et al., 1996), and humans (Meyerhardt et al., 1999).

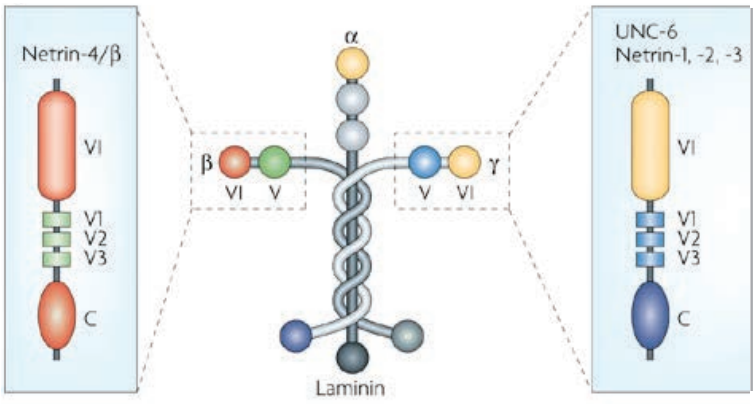


Figure 1.5: Netrin protein structure. The N-terminus of Netrin proteins is composed of domains VI and V, which are homology to laminin. The N-terminus of Netrin 1-3 and NetrinG are most similar to the γ chain of laminin, while Netrin-4 (also known as Netrin-β) is more simialr to the β chain of laminin. The C-domain, also known an NTR domain, does not contain homology to laminin. Figure reproduced with permission from Cirulli and Yebra, 2007.

There are currently six known major vertebrate Netrin proteins (Figure 1.5): Netrins 1-4 and NetrinG1 and 2. Netrins 1-3 are more closely related to the worm and fly Netrins, sharing homology to the laminin γ chain, while Netrin-4 shares more homology with the laminin β chain. Only chicks and fish express Netrin-2 (Serefini et al., 1994; Park et al., 2005). NetrinG1 and 2 are unique in that they have a GPI linkage to the membrane (Nakashiba et al., 2000). Little is known about the cellular functions of the secreted Netrins other than Netrin-1.

Role of Netrin-1 in guidance and migration

One of the best-understood functions of Netrin is its ability to direct growth cones toward their targets by acting as a long-range chemoattractant during neuronal development. The founding member of the Netrin family, Unc6, was discovered as a factor that directs circumferential axon guidance in worms (Hedgecock et al., 1990; Ishii et al., 1992). A conserved role for Netrin proteins in axon guidance was discovered when Netrin-1 and Netrin-2 were isolated from embryonic chick brains as factors capable of promoting outgrowth and axon orientation from spinal cord explants (Serafini et al., 1994; Kennedy et al., 1994). Netrin-1's role as an axon guidance factor *in vivo* was confirmed with the creation of *Netrin-1* mutant mice, which produce severely reduced levels of Netrin-1 and exhibit disrupted commissural axon guidance to the midline in the spinal cord. In fact, multiple commissures in the brain, including the corpus callosum, hippocampal commissure, and anterior commissure, completely fail to form in *Netrin-1* mutant animals (Serafini et al., 1996).

In addition to their chemoattractant roles, Netrin proteins can also repel axons in certain contexts. In *unc6* mutant worms, two distinct guidance defects are observed: guidance of projections ventrally towards the Unc6 source and guidance of projections dorsally away from the Unc6 source (Hedgecock et al., 1990; Ishii et al., 1992). Similarly, Netrin-1 was found to repel trochlear motor neurons from the midline in *in vitro* assays (Colamarino and Tessier-Lavigne, 1995). *In vivo*, however, trochlea motor axon projections are grossly normal in *Netrin-1* hypomorphs and mutant floor plate tissue is still capable of repelling trochlear neurons in *in vitro* assays (Serafini et al., 1996). This suggests either that another chemorepellent is secreted from the floor plate which can compensate for the loss of Netrin-1, or that the small amount of residual Netrin-1 protein is sufficient to repel trochlear motor neurons *in vivo*.

Netrin-1 can act as both a long-range and short-range cue. There is strong evidence that Netrin-1 has long-range guidance activity. The floor plate generates a gradient of Netrin protein that attracts commissural axons from the dorsal spinal cord to cross the ventral midline (Kennedy et al., 2006), and *in vitro* Netrin-1 can influence axonal trajectories at a distance of ~250 μm (Kennedy et al., 1994). On the other hand, during midline crossing in the fly, Netrins have short-range functions. In the absence of Netrins, commissural axons still approach the midline, indicating that long-range guidance is intact. However, the number of commissural axons that successfully crossed the midline is reduced, suggesting a short-range function of Netrins in midline crossing. In support of this, when Netrins are tethered to the cell surface at the midline, commissural crossing is restored (Brankatschk and Dickson, 2006). Interestingly, despite being a secreted protein, most Netrin protein is bound to cell membranes or the

extracellular matrix *in vivo* (Serefini et al., 1994; Kennedy et al., 2006). The significance of this binding is unknown: Netrin substrate binding could simply function to set up and maintain a gradient, or it could be essential for its function as a guidance molecule. Substrate bound Netrin is biologically active and can reorient axons in *in vitro* assays. Furthermore, commissural axon growth cones exert greater forces on Netrin-coated substrates, suggesting that the tension generated by binding to immobilized Netrin may be biologically relevant (Moore et al., 2009).

Netrin proteins signal through a variety of receptors to mediate these diverse effects on axon guidance. In worms, two additional *unc* mutants with unc6-like defects in circumferential axon guidance were described: *unc5* mutants have defects in dorsal circumferential guidance whereas *unc40* mutants have defects in ventral circumferential axon guidance. This led Hedgecock et al. to hypothesize that Unc5 and Unc40 are Unc6 receptors. This proved to be true (Leung-Hagesteijn et al., 1992, Chan et al., 1996), and Unc5 and Unc40 homologues were subsequently found to function as Netrin receptors in vertebrates, as well. Vertebrate Netrin receptors include Unc5A-D (Leonardo et al., 1997), the Unc40 homologue Deleted in Colorectal Cancer (DCC) (Keino-Masu et al., 1996), and the DCC paralogue Neogenin (Vielmetter et al., 1994). Unc40/DCC mediates attraction to Netrin (Keino-Masu et al., 1996), and *DCC* mouse mutants display similar midline guidance defects as *Netrin-1* mutants (Fazeli et al., 1997). The crystal structure of the N-terminus of Netrin-1 bound to DCC and Neogenin shows that Netrin-1 has a rigid, elongated structure with binding sites at opposite ends. A single molecule of Netrin-1 binds to different receptor molecules to form ligand/receptor complexes (Xu et al., 2014). Both *in vitro* and *in vivo* evidence suggests

that Unc5 mediates repulsion from Netrin (Hong et al., 1999). As described above, *unc5* worm mutants have unc6- like defects in midline guidance, whereas in mice, loss of *Netrin-1* encompasses only a subset of the defects present in the various *Unc5* receptor mutants. For example, *Unc5a* null animals have Netrin-1 independent changes in cell death in the spinal cord (Williams et al., 2006) and Unc5b controls vessel branching during vascular development independent of Netrin-1 (Lu et al., 2004). Finally, *Unc5c* mutants have trochlear and phrenic nerve guidance defects, which are absent in *Netrin-1* hypomorphs (Burgess et al., 2006).

In addition to DCC and Unc5 family members, Netrin-1 has also been suggested to signal through other receptors in certain contexts. Down's syndrome Cell Adhesion Molecule (DSCAM) was also reported to be a Netrin-1 receptor involved in midline axon guidance in flies (Andrews et al., 2008). In vertebrates, DSCAM is expressed by commissural axons, can bind Netrin-1, and loss of function studies using siRNAs in mouse suggested that DSCAM mediates turning responses to Netrin-1, both alone and in collaboration with DCC (Ly et al., 2008). However, commissural axons cross the midline normally in *DSCAM* mutant mice (Palmesino et al., 2010), underscoring the importance of studying the role of Netrin-1 and its receptors *in vivo* using carefully controlled conditions.

Role of Netrin-1 in survival

Although best known for its effects on axon guidance, evidence from both development and disease supports a role for Netrin-1 in survival as well. The Netrin receptor DCC was named for its loss of heterozygosity in a majority of colorectal cancer

cases. Though initially proposed to be a tumor suppressor (Fearon et al., 1990), its status is currently controversial since *DCC* mutant mice do not form more tumors, although they do have more aggressive tumor progression in genetic models of colorectal cancer (Fazeli et al., 1997). Despite this, there is clear evidence that DCC and Unc5 function as dependence receptors and trigger cell death in the absence of Netrin-1 binding. For example, expression of DCC in cells that do not normally express this receptor triggers apoptosis, while the addition of Netrin can prevent this cell death (Mehlen et al., 1998).

The mechanism by which unbound DCC causes cell death is not fully understood, although some details of the complex signaling cascade are known. DCC mediated apoptosis requires cleavage of the DCC intracellular domain by caspase-3, which is hypothesized to reveal or release a proapoptotic addiction/dependence domain (Mehlen and Furne, 2005; Forcet et al., 2001; Llambi et al., 2001). DCC mediated cell death also requires caspase-9, which interacts indirectly with the death domain of DCC (Forcet et al., 2001). Netrin may act by blocking caspase-3 cleavage of DCC or by preventing caspase-9 binding to DCC and formation of the caspase-activating complex.

Netrin-1 likely acts through its dependence receptors to regulate tissue renewal *in vivo*. In the intestine, *Netrin-1* is expressed at the bottom of the intestinal crypts while DCC is expressed throughout the epithelium of the intestinal villi. Proliferative cells near the crypt encounter a high concentration of Netrin-1 and are protected from apoptosis. As they differentiate and migrate up the villus, however, they receive less and less Netrin-1, eventually triggering cell death. This process eliminates cells that may have accumulated mutations after undergoing many rounds of proliferation in the harsh

environment of the intestine (Mazelin et al., 2004). Indeed, forced expression of *Netrin-1* throughout the intestinal epithelium causes a decrease in apoptosis and more aggressive tumor progression in colon cancer models (Mazelin et al., 2004).

Tumor cells circumvent this safeguard by down-regulating or inactivating DCC and Unc5 in a variety of cancers (reviewed in Mehlen et al., 2011). Other types of cancer, including breast cancer (Fitament et al., 2008), lung cancer (Delloye-Bourgeois et al., 2009a), pancreatic cancer (Link et al., 2007), and neuroblastoma (Delloye-Bourgeois et al., 2009b) show increased Netrin-1 levels. Autocrine *Netrin-1* expression gives tumor cells a selective advantage at the site of origin and allows them survive while migrating to new environment with little or no Netrin-1 ligand.

Aside from a role in cancer, there is some evidence that Netrin-1 promotes survival during development. Flies express two Netrins: *NetA* and *NetB* (Harris et al., 1996). *NetAB* double knockout animals exhibit the expected defects in commissural axon midline guidance, but also exhibited increased cell death (Newquist et al., 2013). As expected, pan-neuronal expression of *NetA* exacerbated axon guidance defects in double mutants, which supports the long accepted idea that NetA is a tropic factor (Harris et al, 1996; Mitchell et al., 1996), as pan-neuronal expression of NetA appeared to act as a non-directional guidance signal. Unexpectedly, pan-neuronal expression of *NetB* in double mutants reduced apoptosis and rescued the midline guidance defects, even though the reintroduced Netrin signal was not directional. This suggested that NetB acts mainly as a trophic factor to promote survival. Indeed, simply blocking apoptosis was able to rescue the midline guidance defects in *NetAB* double mutants,

providing *in vivo* evidence that the survival and guidance activities of Netrin are intertwined (Newquist et al., 2013).

The role of Netrin-1 as a survival factor in the vertebrate spinal cord is uncertain. One study reported similar number of apoptotic cells in the spinal cord and several other areas of the nervous system at E12 in *Netrin-1* mutants compared to control littermates as assayed by both cleaved caspase-3 and TUNEL staining (Williams et al., 2006). On the other hand, another group reported increased cleaved caspase-3 in spinal cord extracts from *Netrin-1* mutants and observed more TUNEL-positive cells in the spinal cord of E13.5 *Netrin-1* mutants (Furne et al., 2008). *In vitro*, commissural neurons die when cultured, but can survive if provided with Netrin-1. Further, *DCC* mutant neurons can survive *in vitro* without the addition of Netrin-1, suggesting that DCC acts a dependence receptor in commissural neurons (Furne et al., 2008). Thus, the status of Netrin-1 as a vertebrae survival factor *in vivo* remains unresolved.

Role of Netrin-1 in adhesion

Netrins are expressed broadly outside of the developing nervous system and play important roles during tissue morphogenesis (reviewed in Cirulli and Yebra, 2007; Baker et al., 2007). One example is the role of Netrin-1 in adhesion during branching morphogenesis. Multiple secretory tissues, including the pancreas and mammary glands, contain clusters of exocrine cells at the ends of a branching network of epithelial ducts. This complex morphology forms from an initial duct through a process of progressive branching, and Netrin-1 plays a role in this morphogenesis in the pancreas and mammary glands.

The pancreas is a glandular organ that has both endocrine and exocrine functions. During pancreatic development, two primary buds evaginate from the primitive gut epithelium. Interactions with the extracellular matrix and signals from the surrounding mesenchyme promote branching morphogenesis and transform these simple buds into a complex branched ductal system (reviewed in Kim and Hebrok, 2001). Epithelial progenitors of pancreatic endocrine and exocrine cells originate from this ductal system and then migrate into the surrounding mesenchyme and differentiate. Endocrine islet cells secrete hormones including insulin and glucagon into the bloodstream while exocrine acinar cells secrete pancreatic juices important for digestion. Acinar cells are arranged in clusters at the terminal bud of the branched ductal system of the pancreas. Their secretions are carried through this network to the main pancreatic duct, which is connected to the duodenum.

Netrin-1 plays an adhesive role during pancreatic development, where it is expressed by multiple epithelial cell types. Netrin-1 protein localizes to the basement membrane and can bind to type IV collagen, raising the possibility that Netrin-1 plays an adhesive role during pancreatic development. Indeed, pancreatic epithelial cells bind to the C-terminus of Netrin-1 via Integrin α4β6. *In vivo*, integrin α4 and β6 subunits are expressed by cells at sites of Netrin-1 production and/or deposition supporting a role for Integrin α4β6 as an additional Netrin-1 receptor (Yebra et al., 2003).

The mammary glands are another tissue that undergoes extensive branching morphogenesis. During puberty, the terminal end bud, a specialized mammary epithelium tissue, grows from the nipple inward to invade the underlying fat pad, where it branches to form the ductal system. This growth is driven by proliferation of a layer of

cap cells at the tip of the terminal end bulb. Mammary ducts have an outer layer of myoepithelial cells and an inner layer of luminal epithelial cells. As the terminal end bulb invades the fat pad, the cap cells differentiate into the outer myoepithelial cells while the underlying preluminal cells form the inner luminal epithelium. Netrin-1 functions during mammary gland development to maintain adhesion between these two cell layers. Preluminal cells express *Netrin-1* and cap cells express *Neogenin*. In *Netrin-1* or *Neogenin* mutant mammary tissue, these layers fail to adhere, generating an abnormal space between the two cell layers (Srinivasan et al., 2003). In summary, Netrin-1 controls many cellular events during development that could allow it to regulate tissue morphogenesis, including guidance, migration, survival, and adhesion.

The role of Netrin in basement membrane regulation

Evidence from multiple systems supports the idea that Netrin proteins may also directly influence basement membrane regulation, which could contribute to its effects on axon guidance, survival, and morphogenesis. Netrins are capable of binding extracellular matrix components including heparin (Geisbrecht et al., 2003; Kappler et al., 2000), laminin (Schneiders et al., 2007), and type IV collagen (Yerba et al., 2003) and localize to the basement membrane during the morphogenesis of multiple tissues including the lung (Liu et al., 2004), salivary gland (Schneiders et al., 2007), and pancreas (Yerba et al., 2003). Unc6 is required for basement membrane breakdown in the worm, suggesting that basement membrane regulation by Netrins may be an evolutionarily conserved function. During worm development, a uterine “anchor cell” breaches the uterine and vulval basement membranes to connect the two tissues. Unc6

acts through its receptor Unc40 to orient an invasive process towards the basement membrane (Ziel et al., 2009). Unc40 directs this invasive process to fully breach the basement membrane, allowing the anchor cell to invade the vulval tissue (Hagedorn et al., 2013). In *unc6* or *unc40* mutants, the anchor cell fails to cross the basement membrane and the two tissues remain separate (Ziel et al., 2009).

Alternate evidence raises the possibility that Netrin-1 can destabilize the basement membrane in a receptor-independent manner. The first step in basement membrane formation is laminin polymerization (Smyth et al., 1999). Since Netrin-1 is structurally related to laminin, this raises the possibility that Netrin-1 could interfere with laminin polymerization to disrupt basement membrane integrity. Along these lines, Netrin-4, but not Netrin-1, binds to laminin *in vitro* and inhibits laminin polymerization (Schneiders et al., 2007). In sum, while there is accumulating evidence that Netrin-1 can regulate basement membrane integrity, the underlying molecular mechanisms remain unknown.

Netrins play many critical roles in development, with effects not only on axon attraction and repulsion, but also neuronal survival and cell adhesion. Conflicting reports on Netrin-1 function *in vitro* and *in vivo*, for instance the repulsion of trochlear motor neuron axons, limit our understanding of the core functions of Netrin-1. Here, we sought to determine how Netrin-1 mediates its effects in one *in vivo* context: the morphogenesis of the semicircular canals of the inner ear. Using the chick as a model system, we provide new evidence that Netrin-1 functions as a trophic factor during

semicircular canal formation. We then turned to the mouse, where Netrin-1 regulates basement membrane integrity during canal formation. We describe the creation of a conditional *Netrin-1* expression allele and provide evidence that ectopic *Netrin-1* is sufficient to cause excess fusion during canal formation. We also describe the creation of a conditional null allele of *Netrin-1* and provide evidence that complete loss of Netrin-1 protein does not cause more severe fusion defects during canal morphogenesis. It does, however, completely prevent axon guidance to the midline, suggesting that Netrin-1 is required for all commissural axon guidance. These genetic tools will allow future studies to better delineate the many role of Netrin-1 *in vivo.*

Chapter 2: Netrin-1 regulates apoptosis during semicircular canal morphogenesis in chicks

Allison M. Nishitani, Sho Ohta, Tony del Rio, Victoria E. Abraira, Michael Gordon, Donna M. Fekete, Gary C. Schoenwolf, and Lisa V. Goodrich.

A.M. Nishitani performed RCAS injections and all histological and morphological characterizations. S. Ohta performed electroporations. T. del Rio cloned the Netrin-1-myc and GFP RCAS constructs. V.E. Abraira performed pilot experiments demonstrating the gross morphological phenotype of overexpression of Netrin-1 in the chick. M. Gordon performed cell-counting analysis. D.M. Fekete, G.C. Schoenwolf, and L.V. Goodrich made intellectual contributions.

Introduction

The precise orientation of the three semicircular canals (anterior, posterior, and lateral) of the vestibular system underlies the perception of head position. Even slight changes in canal structure cause severe vestibular defects including vertigo, dizziness, and abnormal posture (Sando et al., 2001). In amniote vertebrates, including chicks and mice, formation of the semicircular canals begins when two flat pouches protrude from the dorsal portion of the otic vesicle. The vertical canal pouch forms the anterior and posterior canals while a separate lateral canal pouch forms the lateral canal. In the center of each pouch the opposing epithelial walls of the pouch fuse into a single layer, forming a specialized structure called the fusion plate. Fusion plate cells lose their epithelial morphology and their basement membrane breaks down. The opposing epithelial layers then fuse into a single layer and are cleared, leaving behind the mature canal consisting of an epithelial tube which traces the unfused perimeter of the former canal pouch (Figure 1.4).

Some of the cellular events that drive fusion have been described. In *Xenopus*, localized production of hyaluronan in epithelial cells drives opposing epithelial protrusions together (Haddon and Lewis, 1991). In mice, proliferation of the mesenchyme adjacent to the fusion plate is proposed to provide mechanical force to push the opposing epithelial walls together. *Fgf9* is expressed in the otic epithelium and its receptors *Fgfr1(IIIc)* and *Fgfr2(IIIc)* are expressed in the surrounding mesenchyme. *Fgf9* mutants exhibit failed fusion plate formation, which is attributed to decreased proliferation in the surrounding mesenchyme. Thus, FGF9 from the epithelium signals to the mesenchyme to promote fusion plate formation (Privola et al., 2004). Similarly,

Netrin-1 is expressed at the fusion plate and fusion does not occur in *Netrin-1* mutants, at least in part because of decreased proliferation in the mesenchyme near the fusion plate (Salminen et al., 2000). However, it remains unknown whether this decrease in proliferation is a primary or secondary effect of *Netrin-1* loss.

Removal of fusion plate cells after fusion also appears to occur through different mechanisms across species. Fusion plate clearance could occur via recruitment into the epithelium of the newly formed canal, epithelial to mesenchymal transition, or apoptosis. Although fate-mapping studies are necessary to definitively determine the fate of fusion plate cells, there is evidence that cell death is not used to clear the fusion plate in mice (Martin and Swanson 1993; Nishikori et al., 1999). On the other hand, apoptosis is required for canal formation in chicks (Fekete et al., 1997), demonstrating that different mechanisms for fusion plate clearance are used across species. Many apoptotic cells are present at the fusion plate in the chick, and blocking cell death by overexpressing Bcl2 interferes with fusion plate clearing, resulting in wider diameter canals, or the retention of a canal pouch (Fekete et al., 1997).

As mentioned above, Netrin-1 is required for proper fusion in mice. In *Netrin-1* mutants, fusion arrests: the basement membrane breakdown does not break down at the fusion plate and there is decreased proliferation in the surrounding mesenchyme (Salminen et al., 2000). Further evidence implicating Netrin-1 as an important regulator of canal fusion in mice comes from the study of *Lrig3* mutants. *Lrig3* restricts *Netrin-1* expression to the fusion plate in the lateral canal pouch; in *Lrig3* mutants, *Netrin-1* expression is expanded throughout the lateral canal pouch epithelium. *Lrig3* mutants have a lateral canal truncation as a result of early and ectopic basement membrane

breakdown and fusion throughout the lateral canal pouch. Removing one copy of *Netrin-1* rescues the *Lrig3* lateral canal truncation, demonstrating that increased *Netrin-1* is necessary for the *Lrig3* mutant phenotype (Abraira et al., 2008). Thus, while a loss of *Netrin-1* prevents fusion and canal formation, expanded *Netrin-1* expression causes ectopic fusion and canal truncation. Together, these observations demonstrate that Netrin-1 regulates key cellular events that drive canal formation in mice, although the underlying molecular mechanism remains unknown (Matilainen et al., 2007; Abraira et al., 2008).

Understanding the role of Netrin-1 in any process *in vivo* is complicated by the fact that Netrin-1 controls many cellular events by signaling through several receptors. Further, Netrin can contribute to the same cellular events via different mechanisms. For instance, although Netrin is involved in midline guidance of commissural axons across species, its mechanism of action varies. In flies lacking Netrins, commissural axons approach the midline, but fail to cross. This indicates that long-range guidance is intact and that Netrins function to promote midline crossing at a short-range. Indeed, expressing tethered Netrins at the midline restores midline crossing, demonstrating that Netrins do act as short-range guidance cues in the fly (Brankatschk and Dickson, 2006). In mice, on the other hand, Netrin-1 acts as a long-range guidance cue. In *Netrin-1* mutants, most commissural axon projections are misguided and do not reach the floor plate, and the few axons that project normally to the floorplate cross the midline normally (Serafini et al., 1996). This suggests that Netrin-1 acts as a long-range guidance cue in this system

We used overexpression studies in the developing chick inner ear to determine the main cellular effect of Netrin-1 during canal formation. We found that Netrin-1 is expressed in the fusion plate epithelium in chicks as in mice, but plays a divergent role during canal formation. As described above, in mice Netrin-1 promotes basement membrane breakdown and proliferation of the surrounding mesenchyme (Salminen et al., 2000). Here we show that in chicks Netrin-1 instead regulates cell survival.

Materials and methods

RCAS constructs and virus production

Full-length chick Netrin-1 with a C-terminal myc tag (Serafini et al., 1994) was cloned into ClaI sites of a Replication-Competent Avian Sarcoma retrovirus (RCAS) vector. As a control, the Netrin-1 signal sequence was fused to GFP (ss-GFP) and cloned into the RCAS vector. RCAS virus was produced using chicken fibroblast cells as previously described (Hollenbeck and Fekete, 2003). Virus with titers of at least 2.1x10^8 infectious units were used.

RCAS injections

Fertilized eggs were placed into an incubator at 37°C; this time was designated as time zero. On the morning of the third day of incubation, approximately 5 ml of albumin was removed using a syringe with an 18-gauge needle, and the embryo was allowed to settle away from the top of the eggshell. A small window was then cut in the top of the eggshell to expose the embryo for manipulation. Injections were performed later that day at Hamburger Hamilton (HH) stage 18-19. To aide in visualization of the

embryo, 0.2 cc of India ink (Winsor & Newton) diluted 1:10 in Ringers solution plus penicillin-streptomyecin was injected into the yolk underlying the embryo using a syringe and a 32-gauge needle. A Picospritzer (Parker) attached to a pulled glass capillary was used to inject RCAS virus into the lumen of the otic vesicle. 0.25% Fast Green was added to the virus to allow for visualization of the injection.

Electroporations

Full-length chick Netrin-1 with a C-terminal myc tag (Kennedy et al., 1994) was cloned into an EcoRI site in a modified pCAGGS vector. GFP in the same vector was used as a control. *In ovo* electroporation was performed using HH stage 14-16 chick embryos. Eggs were windowed and plasmid solution (1 µg/µl) containing 0.1% Fast Green to enable visualization was injected into the cavity of the otic vesicle with a fine glass micropipette. Positive and negative electrodes were positioned to achieve electroporation of the dorsal region of the otic vesicle, and five 25 ms pulses at 5 volts were applied using a CUY21 electroporator (NEPA Gene).

Paintfilling

E7 heads were fixed overnight in Bodian fix at 4°C, washed for 10 minutes in 100% ethanol, and dehydrated overnight in 100% ethanol at room temperature. Samples were then rinsed briefly in methyl salicylate and cleared overnight in methyl salicylate at room temperature. Heads were hemisected along the midline and their cochleae were injected from the medial side with White-out (BIC) diluted to 0.025% with methyl salicylate using a pulled glass pipette and a Hamilton syringe.

Immunohistochemistry

Embryonic tissue was fixed overnight at 4°C in 4% PFA/PBS and washed 3 times for 10 minutes in PBS. Tissue was then put through a sucrose series (10% sucrose/PBS, 20% sucrose/PBS, 30% sucrose/PBS) and transitioned into Neg-50 embedding media (Richard-Allen Scientific), with incubation in each solution overnight at 4°C. Tissue was embedded in Neg-50 by freezing with a slurry of dry ice and isopentane.

14 µm cryosections were blocked for one hour at room temperature using 3% BSA in PBS and permeabilized for 15 minutes at room temperature using 1% BSA with 0.1% TritonX-100 in PBS. Sections were incubated overnight at 4°C with the following primary antibodies diluted in the permeabilization solution: 3C2 (1:5, Developmental Studies Hybridoma Bank AMV-3C2), PH3 (1:500, Santa Cruz SC-8656-R), laminin (1:750, Sigma L9393), and myc (1:200, Santa Cruz 56633). The following day, sections were incubated in secondary antibody diluted in permeabilization solution for 1.5 hours at room temperature (Alexa Fluor 488, 568, or 647 1:1500, Life Technologies). Sections were counterstained with DAPI (1:10,000) and mounted using Fluoromount-G mounting media (Southern Biotech).

For double labeling with TUNEL and laminin, immunostaining was performed as described above up to the addition of the secondary antibody. Slides were then post-fixed in 1% PFA/PBS for 15 minutes and permeabilized for 10 minutes in 0.5% TritonX-100 before proceeding with the ApopTag fluorescent TUNEL kit (Millipore).

Cell counting

Images (1392 x 1040 pixels) were acquired of tissue sections stained for PH3 and DAPI from wild type, GFP only control, and Netrin-1 overexpressor ears before and during fusion. 200 x 100 pixel boxes, similar in size to those used to quantify proliferation in the mesenchyme in mice (Salminen et al., 2000), were manually placed using the DAPI channel to define regions of interest for cell counting: the first two boxes flanked the fusion plate and a third box was placed more medially. ImageJ software was used to count the number of PH3 positive cells present in the regions specified by boxes. The cell counts were grouped into twelve groups: wild type pre-fusion (box 1-3), wild type fusing (box 1-3), Netrin-1 overexpressor (box 1-3), and GFP only control (box 1-3). Each group was compared against every other group using a one-way analysis of variance.

Results

The fusion plate is a transient structure, thus embryonic staging is critical in studying the cellular events that occur during fusion. While this is relatively difficult in mice, developing chicks can be staged and manipulated individually. The majority of canal morphogenesis occurs between E6 and E7 (HH stage 29-30) in the chick (Figure 2.1 A). By E6, the auditory and vestibular components of the inner ear are discernable: the vertical and lateral canal pouches which will give rise to the semicircular canals are visible, fusion plates have begun to form in the center of the canal pouches, and the cochlea has begun to grow out. By E7, the cells of the fusion plate have been removed to form the semicircular canals.

Cell counting

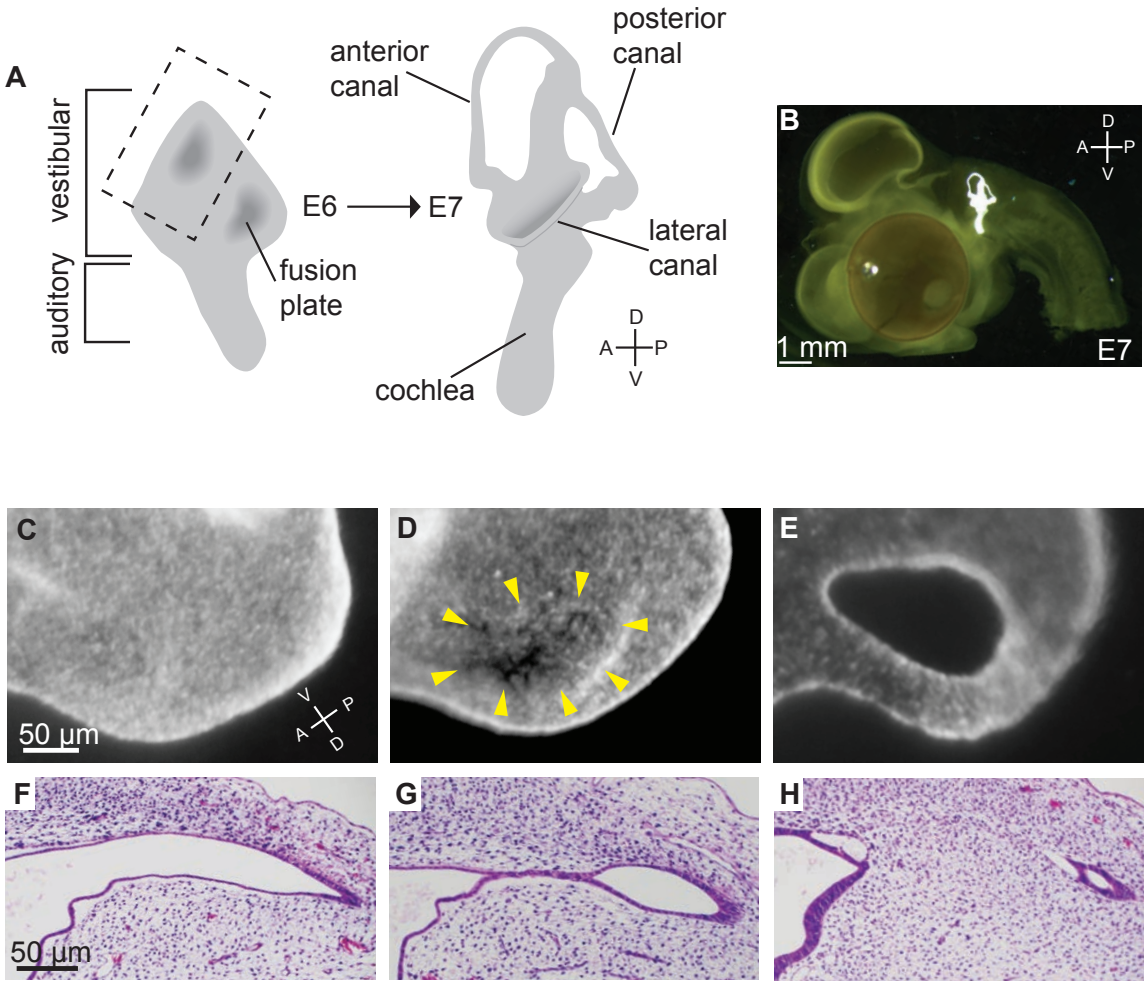
Images (1392 x 1040 pixels) were acquired of tissue sections stained for PH3 and DAPI from wild type, GFP only control, and Netrin-1 overexpressor ears before and during fusion. 200 x 100 pixel boxes, similar in size to those used to quantify proliferation in the mesenchyme in mice (Salminen et al., 2000), were manually placed using the DAPI channel to define regions of interest for cell counting: the first two boxes flanked the fusion plate and a third box was placed more medially. ImageJ software was used to count the number of PH3 positive cells present in the regions specified by boxes. The cell counts were grouped into twelve groups: wild type pre-fusion (box 1-3), wild type fusing (box 1-3), Netrin-1 overexpressor (box 1-3), and GFP only control (box 1-3). Each group was compared against every other group using a one-way analysis of variance.

Results

The fusion plate is a transient structure, thus embryonic staging is critical in studying the cellular events that occur during fusion. While this is relatively difficult in mice, developing chicks can be staged and manipulated individually. The majority of canal morphogenesis occurs between E6 and E7 (HH stage 29-30) in the chick (Figure 2.1 A). By E6, the auditory and vestibular components of the inner ear are discernable: the vertical and lateral canal pouches which will give rise to the semicircular canals are visible, fusion plates have begun to form in the center of the canal pouches, and the cochlea has begun to grow out. By E7, the cells of the fusion plate have been removed to form the semicircular canals.

Figure 2.1: Canals form through a fusion process in the chick. A) The majority of canal morphogenesis occurs between E6 and E7 in the chick. At E6, the two major compartments of the mature inner ear are discernable, with the dorsal part of the inner ear developing into the vestibular system and the ventral part developing into the auditory system. Dorsally, the vertical and lateral canal pouches have grown out from the otic vesicle and fusion plates are beginning to form (gray shading). By E7, fusion has occurred and the canal pouches have resolved into the mature anterior, posterior, and lateral semicircular canals of the vestibular system. Ventrally, the cochlea of the auditory system has grown out. B) The inner ear is visible within the E7 chick head when injected with white paint through a technique known as paintfilling. C-F) The region of the vertical canal pouch outlined in a dashed box in (A) is shown in E6-E6.25 paintfilled ears. F-G) H&E staining on tissue sections through the E6-E6.25 anterior canal demonstrates some of the cellular changes that accompany fusion. Canal formation starts with a canal pouch (C) with the opposing epithelial walls of the pouch separated by a visible fluid filled space (F). Next, a fusion plate forms in the center of the pouch (arrowheads, D) where the opposing epithelial walls come together and fuse into a single layer (G). Finally, the fusion plate is cleared (E). The edges of the fusion plate form an intact epithelium and eventually mesenchymal cells occupy the area where the fusion plate was (H).

Figure 2.1 (continued)



To visualize canal morphogenesis at the gross level, white paint can be injected into the inner ear, a technique known as paintfilling. Wild type specimens between E6-E6.25 were paintfilled and the anterior canal pouches from specimens at different stages in canal morphogenesis are shown (Figure 2.1 C-E). Before fusion begins, the opposing epithelial walls of the canal pouch are separated by a visible fluid filled space (Figure 2.1 F). Next, a fusion plate forms in the center of the canal pouch (arrowheads, Figure 2.1 D) where the opposing epithelial walls come together and fuse into a single layer (Figure 2.1 G). Finally, the fusion plate is cleared leaving the mature canal (Figure 2.1 E). The cells at the edge of the fusion plate reform an intact epithelial tube and the mesenchymal cells occupy the location of the former fusion plate (Figure 2.1 H).

In mice, the axon guidance factor *Netrin-1* is specifically expressed at the fusion plate (Salminen et al., 2000) and expanded *Netrin-1* expression throughout the canal pouch epithelium results in early and ectopic fusion (Abraira et al., 2008). Chicks have two Netrin family members, Netrin-1 and Netrin-2, whose combined expression equals that of *Netrin-1* expression in mammals, which lack *Netrin-2*. *Netrin-1* and *Netrin-2* are expressed at the fusion plate in chicks (Abraira et al., 2010), similar to *Netrin-1* expression at the mouse fusion plate (Salminen et al., 2000), suggesting that Netrins may play a conserved role during fusion. Interestingly, in mice *Netrin-1* is expressed in the epithelial cells that will form the fusion plate shortly after formation of the otic vesicle, at E10.5 (Salminen et al., 2000; Matilainen et al., 2007), even though fusion does not occur until around E12.5. The role of Netrin-1 in the otic epithelium at these earlier stages is unknown. To determine if the precise spatiotemporal patterns of *Netrin-1* and *Netrin-2* expression in the developing chick inner ear match *Netrin-1* expression

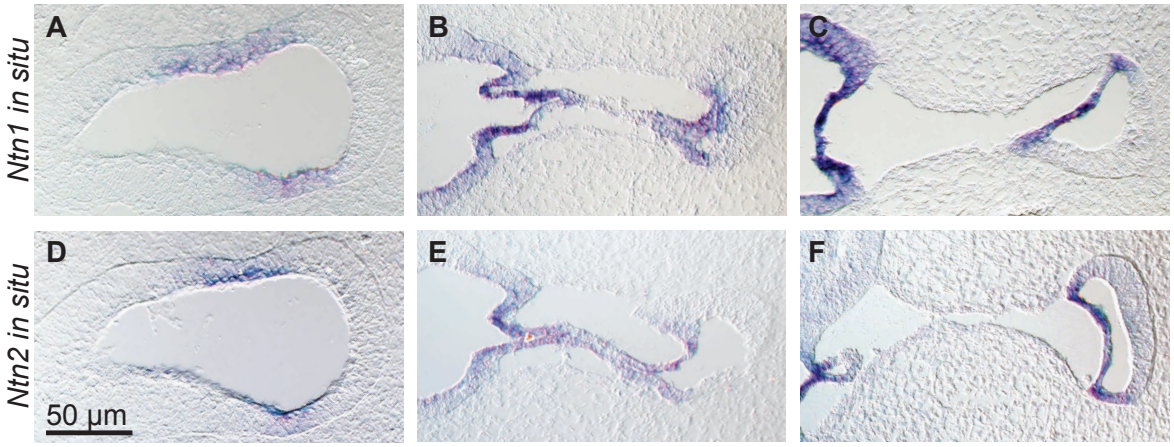


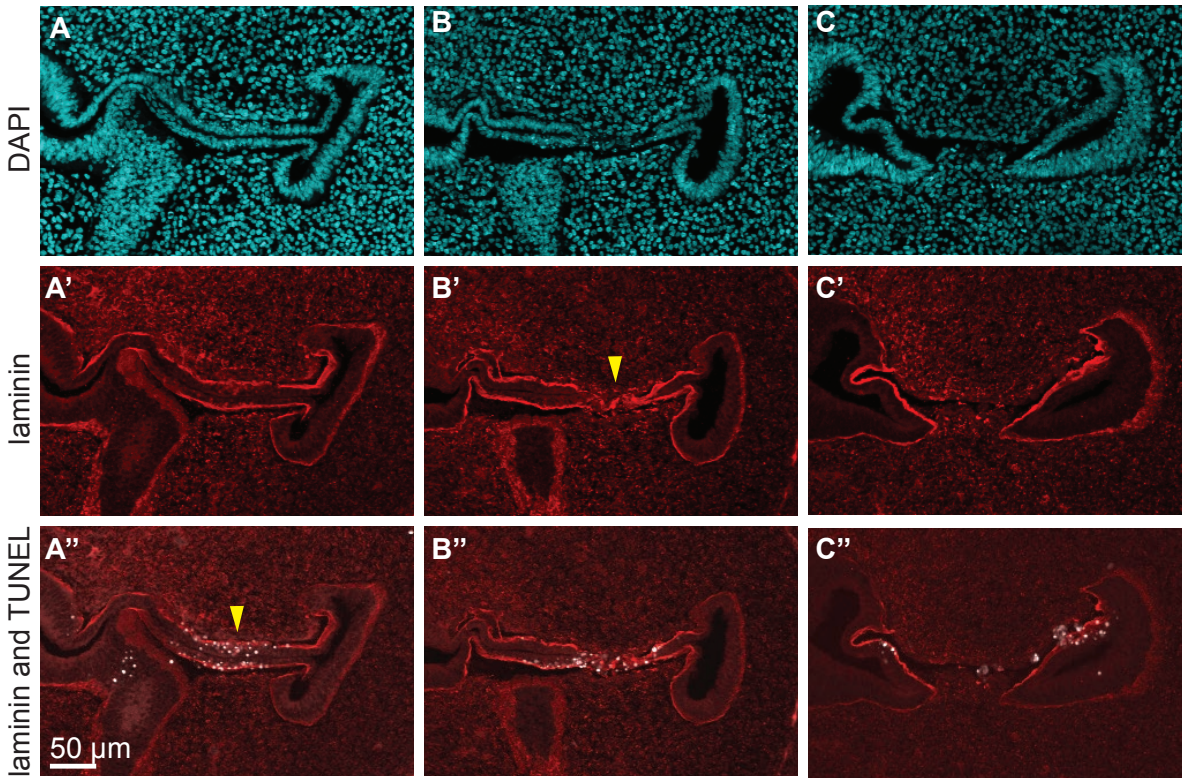
Figure 2.2: Netrins are expressed in the otic epithelium and poised to regulated fusion. *In situs* on tissue sections through the future posterior canal at E5 and E6-E6.25. A-C) *In situs* against chick *Ntn1*. D-F) *In situs* against chick *Ntn2*. *Ntn1* (A-C) and *Ntn2* (D-F) have similar expression in the otic epithelium. Both *Netrins* are expressed in the cells that will form the fusion plate from an early age (A and D, E5 shown). Both *Netrins* are expressed at the fusion plate (B and E) and continue to be expressed by the epithelial cells of the inner canal rim after fusion is complete (C and F).

in the mouse, we used *in situ* hybridization to determine *Netrin-1* and *Netrin-2* expression patterns before, during, and after fusion. We confirmed that both *Netrin-1* and *Netrin-2* are expressed at the fusion plate (Figure 2.2 B and E), and also showed that *Netrin-1* and *Netrin-2* continue to be expressed in the inner canal rim after fusion is complete (Figure 2.2 C and F), similar to what has been reported for *Netrin-1* in mice (Salminen et al., 2000). We also examined *Netrin-1* and *Netrin-2* expression earlier in the developing inner ear at E3, E3.5, E4, E4.5 and E5. *Netrin-1* and *Netrin-2* are both expressed early in the otic epithelium, with expression detectable in the regions that will form the fusion plate as early as E3.5 (Figure 2.2 A and D, E5 shown). In chicks, the otic vesicle forms around E3 and fusion occurs around E6.25, suggesting that Netrins play a role in early inner ear development before fusion. Additionally, the expression patterns of *Netrin-1* and *Netrin-2* in chicks are similar to *Netrin-1* in mice (Salminen et al., 2000), raising the possibility that Netrin plays a conserved role in canal morphogenesis between mice and chicks.

To determine the main cellular effect of Netrin-1 during fusion in the chick, we first sought to establish the normal timing of the cellular events that transform the canal pouch into an epithelial tube. While apoptosis (Fekete et al., 1997; Lang et al., 2000) and basement membrane breakdown (Kobayashi et al., 2007) have both been reported at the fusion plate in chicks, the precise temporal relationship between these two cellular events has not been examined. Detachment from the basement membrane can trigger apoptosis, an event called anoikis. We therefore wondered whether cell death at the fusion plate could be a result of basement membrane breakdown. We used the TUNEL assay to label apoptotic cells and laminin to label the basement membrane

Figure 2.3: Apoptosis precedes basement membrane breakdown during the fusion process. Immunostaining of tissue sections through the posterior canal before, during, and after fusion (E6-E6.25) are shown. A-C) DAPI (cyan) marks cell nuclei. A'-C') laminin (red) stains the basement membrane. A''-C'') laminin (red) identifies the basement membrane and TUNEL (white) identifies apoptotic cells. Just before fusion begins the opposing epithelial walls are aligned but separate (A). At this stage, laminin staining shows that the basement membrane separating the epithelium from the underlying mesenchyme is intact (A'). TUNEL staining shows that apoptosis is detectable in the epithelial cells that will form the fusion plate (arrowhead A''). Fusion occurs when the opposing epithelial layers fuse into a single layer in the middle of the canal pouch (B). Laminin staining shows that the basement membrane breaks down at the fusion plate (B' arrowhead). TUNEL staining shows widespread cell death at the fusion plate (B''). When fusion is complete, the epithelial cells that had occupied the center of the canal pouch are gone and the mature epithelial tube of the canal is formed (C). Laminin staining shows that the basement membrane reforms around the inner rim of the new canal epithelium (C'). TUNEL staining reveals limited cell death at the inner rim of the newly formed canal epithelium (C'').

Figure 2.3 (Continued)



before, during, and after fusion between E6-E6.25 in wild type chick tissue sections. Similar to previous reports (Fekete et al., 1997), we observed widespread cell death at the fusion plate (Figure 2.3 B'') and some residual cell death in the newly formed inner canal rim after the completion of fusion (Figure 2.3 C''). The cell death present at the fusion plate (Figure 2.3 B'') appears to overlap precisely with the *Netrin-1* expression domain (Figure 2.2 B) although performing *in situ* hybridization for both cell death and *Netrin-1* on the same tissue is necessary to confirm this. We also observed a significant amount of cell death in the epithelial cells that will form the fusion plate well before the opposing epithelial layers fuse (Figure 2.3 A''), similar to previous reports (Lang et al., 2000). This pre-fusion cell death precedes the breakdown of the basement membrane at the fusion plate (Figure 2.3 A''), suggesting that apoptosis at the fusion plate is not a result of anoikis.

Another cellular mechanism that has been implicated in canal formation in mice is proliferation of the mesenchyme adjacent to the fusion plate. Mesenchymal proliferation is hypothesized to provide a mechanical force to push the opposing epithelial layers together during fusion plate formation. Both *Fgf9* and *Ntn1* mutant animals have defects in fusion that have been at least partially attributed to decreased mesenchymal proliferation (Pirvola et al., 2004; Salminen et al., 2000). We examined cell proliferation in wild type chick tissue sections before, during, and after fusion by staining for PH3 between E6-E6.25. ImageJ software was used to count PH3 positive cells in defined areas surrounding the fusion plate (Figure 2.4). The number of dividing cells in the mesenchyme surrounding the fusion plate did not differ across the

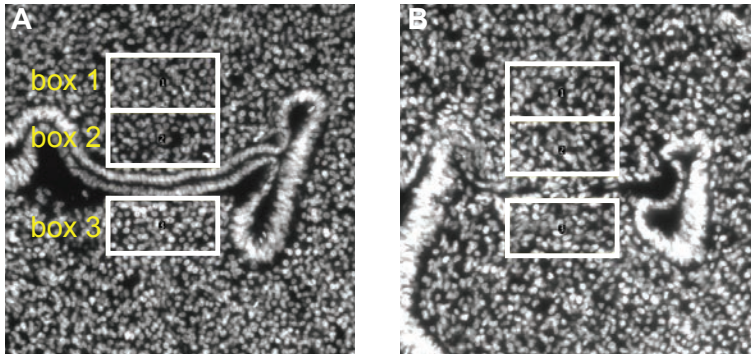


Figure 2.4: Box placement for cell counting. Representative examples of box placement on the DAPI channel are shown on tissue sections both before (A) and after (B) fusion. After the regions of interest has been manually placed on the DAPI channel, ImageJ software was used to count the number of PH3 positive cells within the specificed boxes.

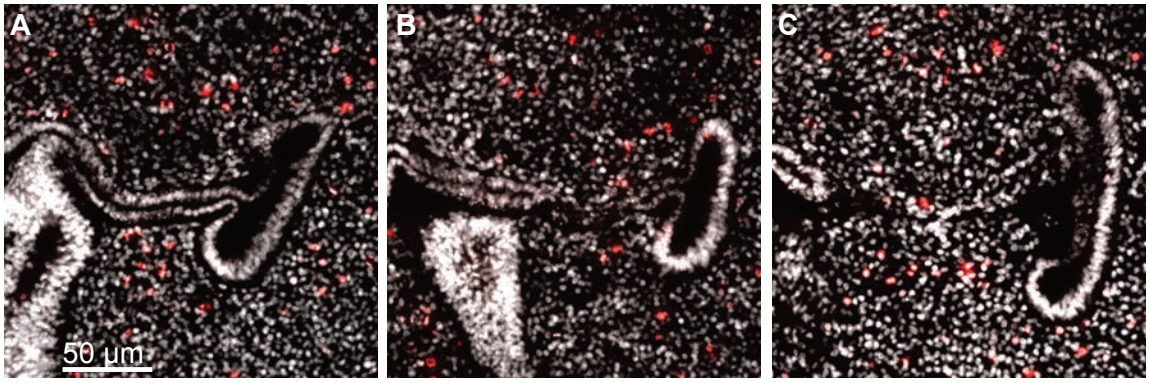


Figure 2.5: Mesenchymal cell division appears uniform throughout the fusion process. Immunostaining of tissue sections through the posterior canal before, during, and after fusion (E6-E6.25) are shown. A-C) DAPI (white) identifies nuclei and PH3 (red) identifies mitotic cells. PH3 staining just before (A), during (B), and after fusion (C) shows fairly uniform proliferation in the mesenchyme and very little proliferation in the epithelium.

Table 2.1: Quantification of cell division in wild type mesenchyme. PH3 immunostaining of tissue sections through the posterior canal before, during, and after fusion (E6-E6.25) was used to identify mitotic cells. As shown in Figure 2.3, regions of interest were defined manually using the DAPI channel, and ImageJ software was used to count the number of PH3 positive cells within the specified boxes. The cell counts were grouped into twelve groups: wild type aligned (box1-3) and wild type fused (box 1-3) [shown in Table 2.1] and Netrin-1 overexpression (box 1-3) and GFP control (box 1-3) [shown in Table 2.2]. Each group was compared against every other group using a one-way analysis of variance. There was not a statistically significant difference in the mean number of PH3 positive cells across any of the groups as the p-value (0.386) was greater than 0.05.

Group	Sample size (number of tissue sections)	Mean number of PH3 positive cells per section
Wild type aligned: box 1	100	3.05
Wild type aligned: box 2	100	2.4
Wild type aligned: box 3	100	2.63
Wild type fused: box 1	16	2.44
Wild type fused: box 2	16	2.56
Wild type fused: box 3	16	2.56

developmental stages examined (Figure 2.5, Table 2.1), suggesting that the increase in mesenchymal proliferation observed in mice may not occur in chicks.

Based on the early expression of *Netrin-1* and *Netrin-2* far in advance of fusion, we hypothesized that Netrins direct early fusion plate formation by regulating apoptosis and/or basement membrane breakdown at the chick fusion plate. To determine if Netrin-1 is sufficient to affect when and how fusion progresses in the chick, we first used RCAS, a replication competent avian retrovirus, to overexpress *Netrin-1* in the developing inner ear. A C-terminal myc tag was added to Netrin-1 due to the lack of effective Netrin antibodies for immunohistochemistry. This myc-tagged Netrin-1 has been shown to be biologically active in *in vitro* axon outgrowth assays (Serafini et al.,1994). Netrin-1-myc RCAS was injected into the lumen of the newly formed chick otic vesicle at E3 and tissue was harvested at E6.25 for histology or E7 to assess gross canal morphology by paintfilling.

Staining for 3C2, a viral coat protein, shows infection of the otic epithelium (Figure 2.6 A). As expected, infection was variable across animals, ranging from complete infection of the epithelium, as shown in Figure 2.6 A, to very sparse salt and pepper infection of the epithelium. Infection of the mesenchyme was also observed and varied across animals from very dense to very sparse. Staining for myc showed that the Netrin-1-myc fusion protein localized to the basement membrane (Figure 2.6 B), which was labeled by laminin staining (Figure 2.6 C).

After confirming the expression of Netrin-1-myc after RCAS injection, we examined the consequences of *Netrin-1* overexpression on canal morphogenesis at a gross level by paintfilling. Netrin-1-myc or GFP RCAS were injected into one of the

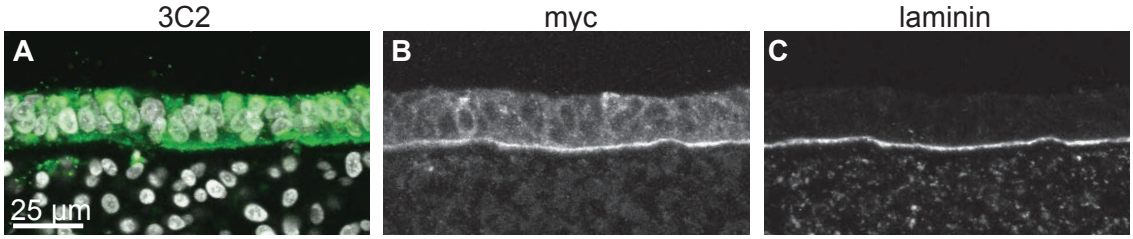


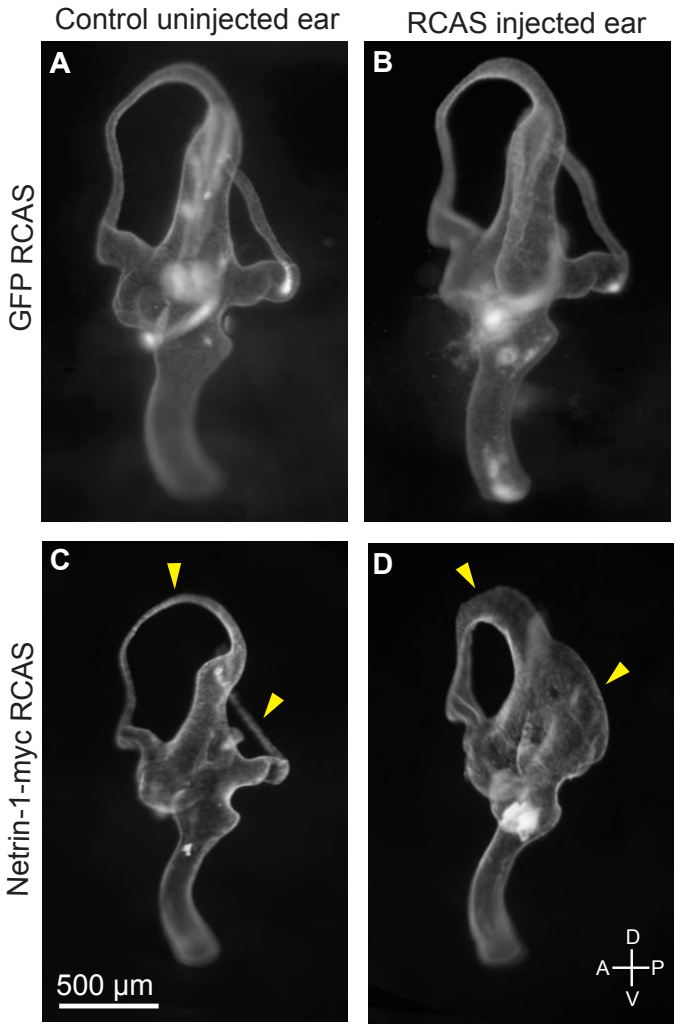
Figure 2.6: RCAS mediated overexpression of Netrin-1-myc leads to incorporation of fusion protein at the basement membrane. Replication competent RCAS virus carrying a myc-tagged version of chick Netrin-1 was injected into the lumen of the newly formed chick otic vesicle at E3. Immunostaining of tissue sections at E6.25 are shown. A) 3C2 staining (green) against a viral coat protein identifies infected cells. B) Myc staining detects the Netrin-1-myc fusion protein. C) Laminin staining detects the basement membrane. RCAS virus leads to infection of the otic epithelium (A). The Netrin-1-myc fusion protein (B) colocalizes to the basement membrane (C).

chick's otic vesicles at E3. Both of the animal's inner ears were paintfilled at E7 and the morphology of the virus-injected inner ear was compared to the control uninjected inner ear. GFP RCAS did not affect canal formation (Figure 2.7 A and B, n=0/19 GFP RCAS ears affected). On the other hand, when Netrin-1-myc RCAS was injected, canal formation was disrupted in the virus-injected ear compared to the uninjected control ear of the same animal (Figure 2.7 C vs. D, n=20/25 Netrin-1-myc RCAS ears affected). Surprisingly, rather than accelerating fusion, overexpression of *Netrin-1* instead prevented fusion. We observed a range of phenotypes, likely due to variability in the extent of infection across animals, that spanned from a complete absence of fusion resulting in canal pouches still being present at E7 (see posterior canal in Figure 2.7 D) to an arrest or delay in fusion resulting in fat canals (see anterior canal in Figure 2.7 D). Failed or delayed fusion is the opposite of the canal truncation defect observed in mice with expanded *Netrin-1* expression (Abraira et al., 2008), but is strikingly similar to the phenotype observed when apoptosis was blocked using Bcl2 RCAS (Fekete et al., 1997). This suggested that the main cellular effect of Netrin-1 in the developing chick inner ear is to regulate apoptosis at the fusion plate, an event that does not seem to occur in mice (Martin and Swanson 1993; Nishikori et al., 1999). Thus, Netrin-1 may play divergent roles in canal formation.

Since RCAS-mediated *Netrin-1* overexpression sometimes resulted in partially fused fat canals (see anterior canal in Figure 2.7 D), it is possible that the main effect of Netrin-1 overexpression was to prevent efficient clearing of fusion plate cells. As described, RCAS- mediated overexpression of Bcl2 gave a strikingly similar phenotype at the gross level with some canal pouches remaining entirely unfused and some canal

Figure 2.7: RCAS mediated overexpression of Netrin-1-myc interferes with the fusion process. Paintfills at E7 reveal the gross morphology of the chick inner ear. A-B) As a control, RCAS carrying GFP fused to the Netrin-1 signal sequence was injected at E3 into the lumen of one of the chick's otic vesicles. At E7, there was no difference in the gross morphology of the uninjected control ear (A) compared to the GFP RCAS injected ear (B) (n=0/19 GFP RCAS ears affected). C-D) When RCAS carrying Netrin-1-myc was injected at E3 into the lumen of one of the chick's otic vesicles, at E7 the uninjected control ear displayed a normal morphology (C) while the Netrin-1 RCAS injected ear often displayed fusion defects (arrowheads D) compared to the control ear from the same animal (n=20/25 Netrin-1-myc RCAS ears affected). Both a complete failure of fusion (see posterior canal in D) and an arrest or delay in fusion resulting in a fat canal (see anterior canal in D) were observed.

Figure 2.7 (Continued)



pouches partially fusing to form fat canals. In these animals, abnormally long fusion plates were observed where the opposing epithelial layers had come together, but had not fused and been cleared (Fekete et al., 1997). We therefore looked at histological sections at E6.25, the stage when fusion normally occurs, to determine whether overexpression of *Netrin-1* interferes with fusion plate formation or fusion plate clearing. H&E staining showed that while fusion was either in progress or complete in control uninjected ears (Figure 2.8 A and C), the Netrin-1-myc RCAS injected ears from the same animals had not formed fusion plates (Figure 2.8 B and D). Thus, Netrin-1 appears to act early to enable fusion plate formation in the chick. We then set out to examine the cellular events that normally occur during fusion in the chick to determine how overexpression of *Netrin-1* might be preventing fusion plate formation. However, since the fusion process occurs very quickly, we first wanted to find another method to overexpress Netrin-1-myc that gave a more penetrant phenotype. To this end, we turned to *in ovo* electroporation of a Netrin-1-myc overexpression construct.

CAG promoter driven Netrin-1-myc and GFP were co-electroporated into the dorsal otic epithelium of the right ear of HH stage 14-16 embryos and tissue was harvested at E6.25 for histology or at E7 to assess gross canal morphology by paintfilling. GFP was also electroporated alone as a control. Immunohistochemistry on E6.25 tissue sections showed that almost every cell in the canal epithelium had been electroporated, as seen by GFP expression (Figure 2.9 A). This high efficiency of electroporation was observed in all 6 Netrin-1-myc electroporated samples examined by immunohistochemistry (data not shown). Staining for myc showed that the Netrin-1-myc

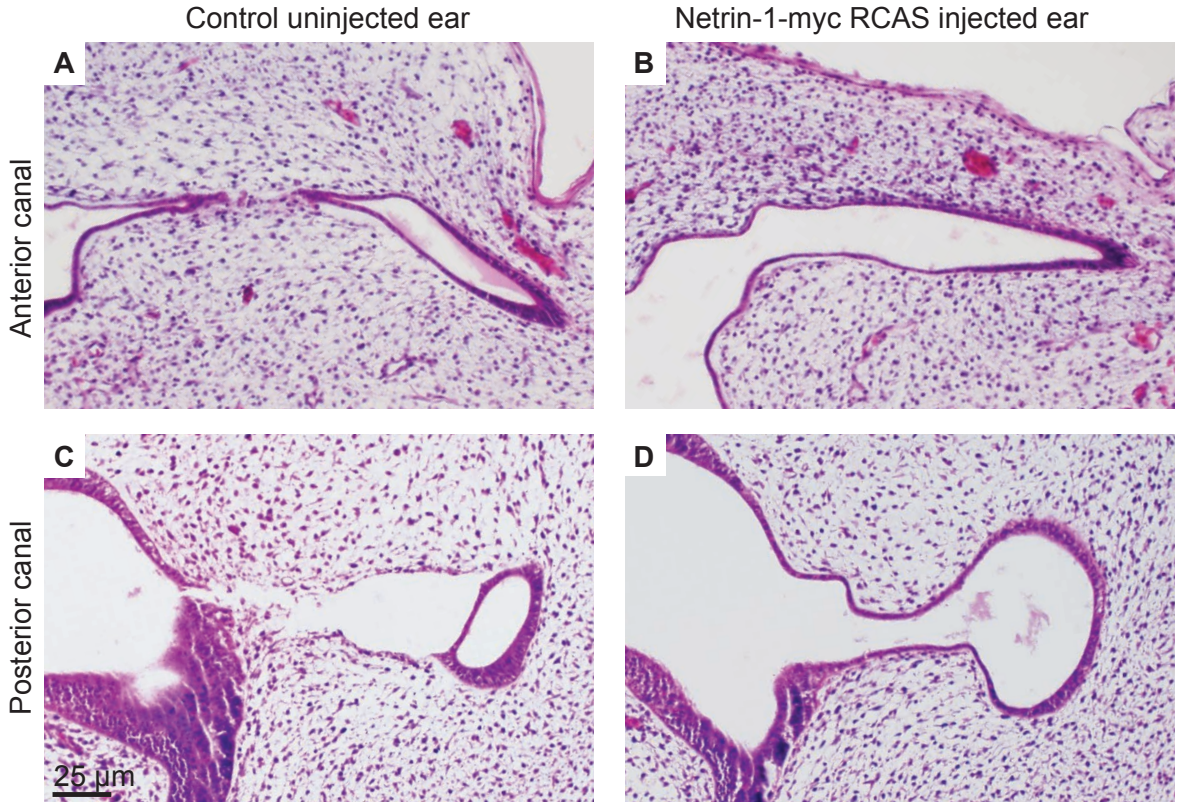


Figure 2.8: RCAS mediated overexpression of Netrin-1-myc interferes with fusion plate formation. H&E staining of E6.25 tissue sections through the anterior (A-B) or posterior (C-D) canal are shown. RCAS carrying Netrin-1-myc was injected at E3 into the lumen of one of the chick's otic vesicles, and tissue was collected at E6.25, when the fusion process occurs. Overexpression of Netrin-1-myc interferes with fusion plate formation. For example, in an animal where the uninjected control anterior canal had formed a fusion plate (A), the otic epithelium of the Netrin-1-myc RCAS injected ear had not yet fused (B). Similarly, in an animal where the uninjected control posterior canal had already completed the fusion process (C), the otic epithelium of the Netrin-1-myc RCAS injected ear had still not fused (D).

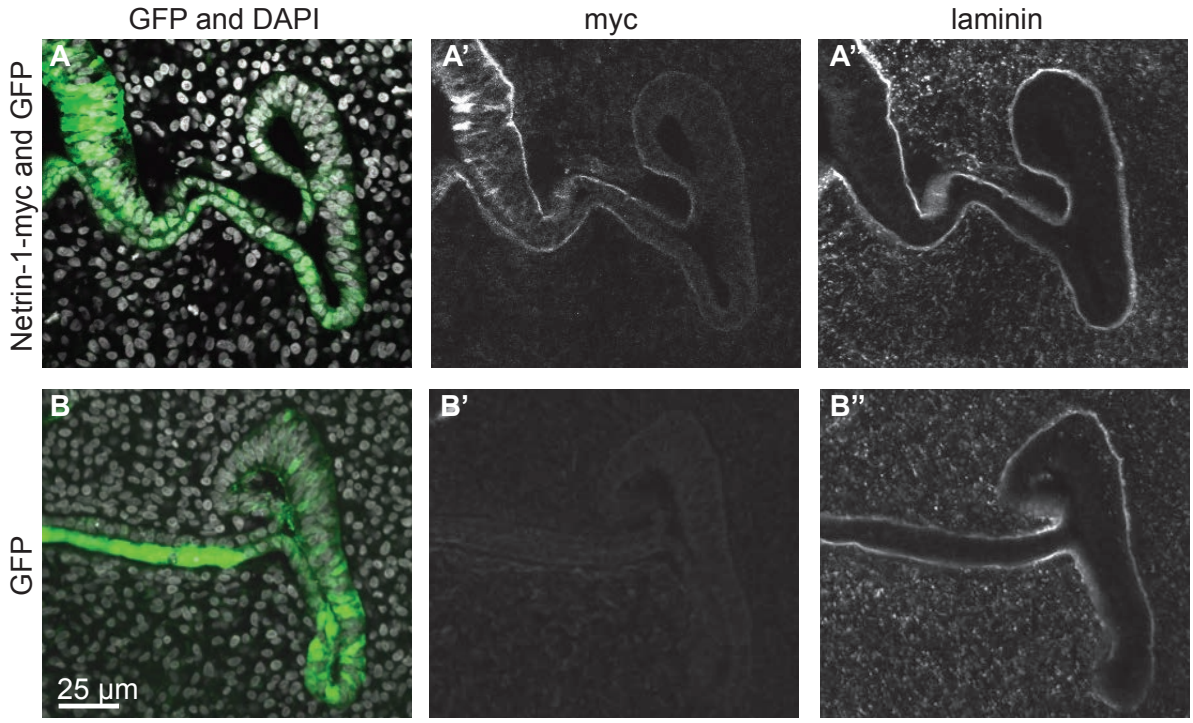


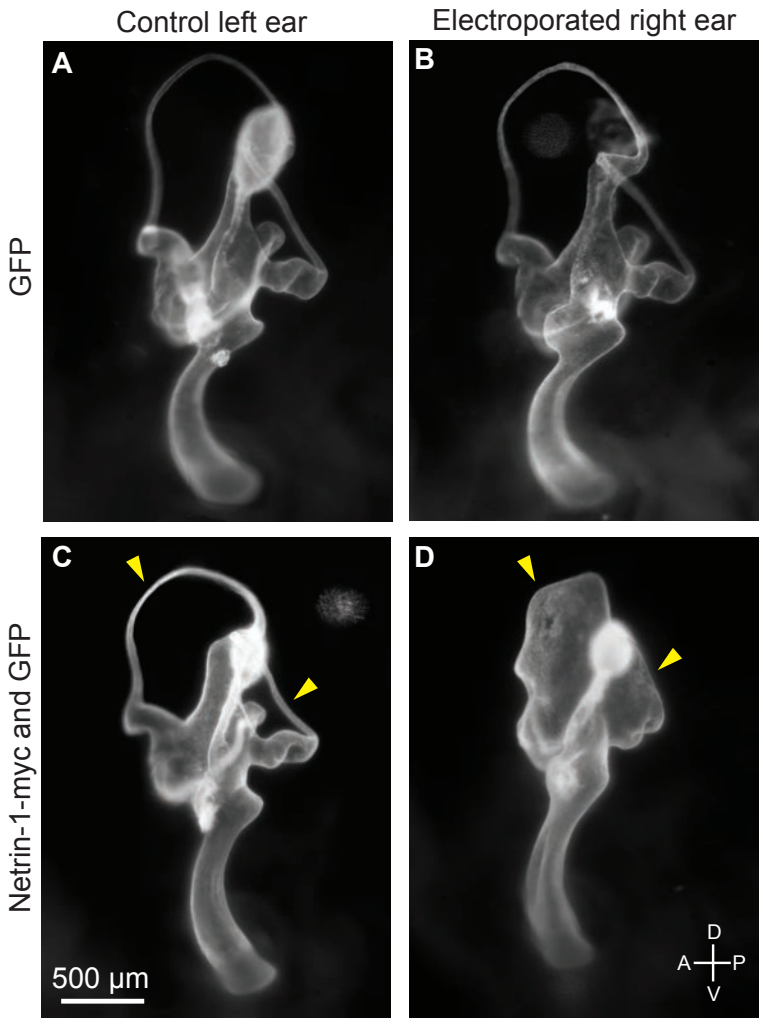
Figure 2.9: Electroporation mediated overexpression of Netrin-1-myc leads to incorporation of fusion protein at the basement membrane. CAG promoter driven Netrin-1-myc and GFP were coelectroporated into the right otic vesicle at HH stage 14-16. GFP was electroporated alone as a control. Immunostaining of tissue sections through the posterior canal at E6.25 are shown. A-B) DAPI (white) identifies cell nuclei and GFP (green) identifies electroporated cells. A'-B') Myc staining detects the Netrin-1-myc fusion protein. A''-B'') Laminin staining detects the basement membrane. GFP shows very efficient electroporation of nearly every cell in the otic epithelium (A and B). The Netrin-1-myc fusion protein (A') colocalizes to the basement membrane (A'').

fusion protein localized to the basement membrane (Figure 2.9 A'), which was labeled by laminin staining (Figure 2.9 A'').

After confirming the expression of Netrin-1-myc after electroporation, we examined gross canal morphology by paintfilling at E7. As a control, chicks were electroporated with GFP alone in one otic vesicle. In both cases, the morphology of the electroporated inner ear was compared to the control unelectroporated inner ear. Electroporation of GFP alone did not affect canal formation (Figure 2.10 A and B, n=0/6 GFP alone ears affected). In contrast, when Netrin-1-myc was co-electroporated with GFP, canal formation was disrupted in the electroporated ear compared to the unelectroporated control ear (Figure 2.10 C vs. D, n=8/8 Netrin-1-myc electroporated ears affected). Electroporation of Netrin-1-myc interfered with fusion, with phenotypes ranging from a complete absence of fusion resulting in canal pouches still being present at E7 to an arrest or delay in fusion resulting in fat canals. While this phenocopied Netrin-1-myc RCAS overexpression, the phenotypes observed after electroporation were more penetrant and more severe. The canal phenotype after electroporation of Netrin-1-myc was 100% penetrant, compared to 80% penetrance for the Netrin-1-myc RCAS injections. This is likely because electroporation targeted almost every cell in the canal epithelium (Figure 2.9 A). Further, the canal defects observed with electroporation of Netrin-1-myc were more severe. In Netrin-1-myc RCAS injected ears, 20% of canals were severely affected and remained unfused (n=15/75) compared to 67% of canals remaining unfused after electroporation of Netrin-1-myc (n=16/24). For these reasons, we used electroporation to overexpress Netrin-1-myc for all remaining experiments.

Figure 2.10: Electroporation mediated overexpression of Netrin-1-myc interferes with the fusion process. Paintfills at E7 reveal the gross morphology of the chick inner ear. A-B) As a control, GFP alone was electroporated at HH stage 14-16 into the right otic vesicle. At E7, there was no difference in the gross morphology of the control left ear (A) compared to the GFP electroporated right ear (B) (n=0/6 GFP ears affected). C-D) At E7 when the control ear displayed a normal morphology (C), the Netrin-1-myc electroporated ear displayed fusion defects (arrowheads D, n=11/11 Netrin-1 electroporated ears affected). Both a complete failure of fusion (see anterior and posterior canals in D) and an arrest or delay in fusion resulting in a fat canal were observed. Since the electroporation technique resulted in a 100% penetrant phenotype, this method of Netrin-1-myc overexpression was used for the rest of the analysis.

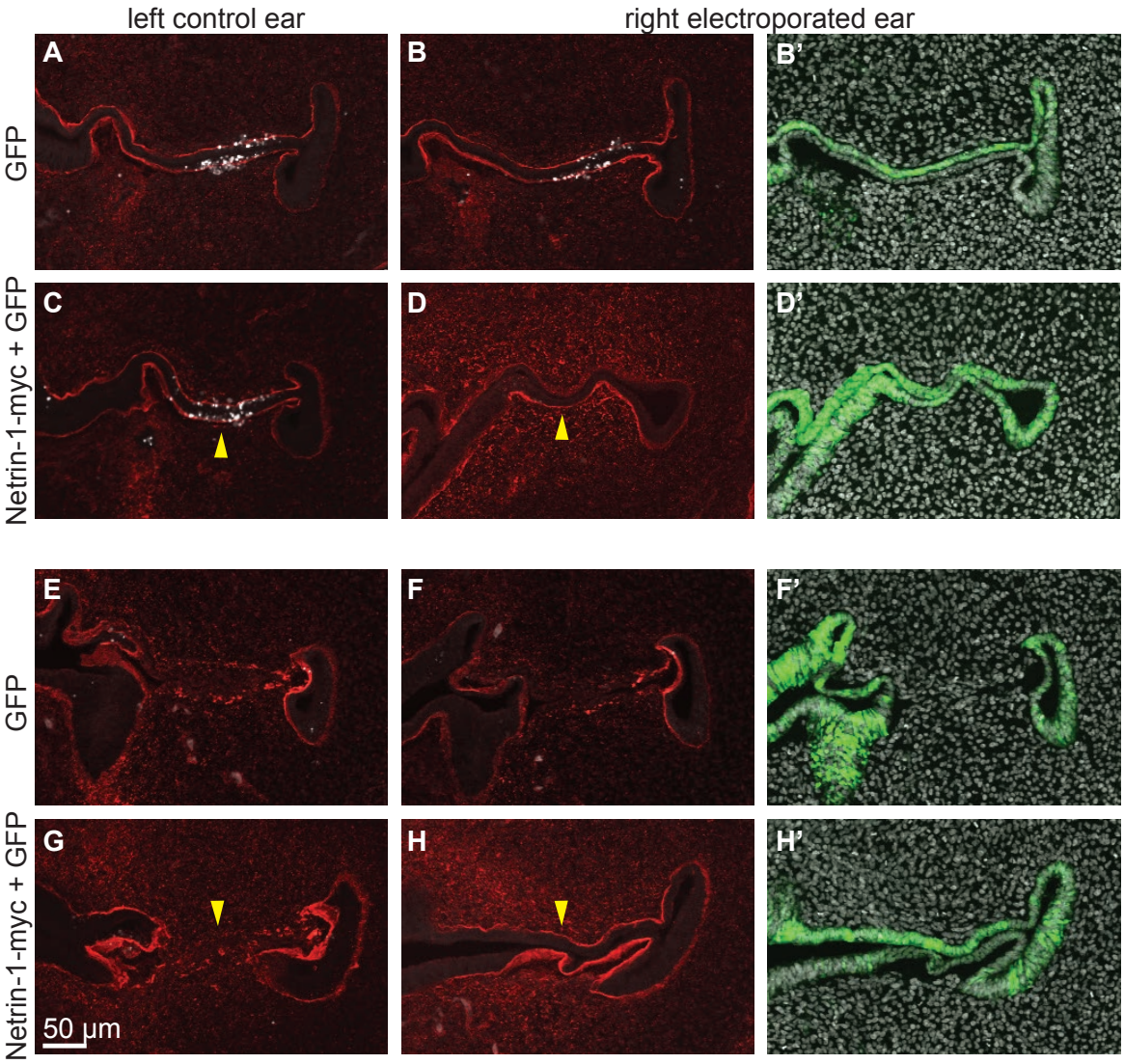
Figure 2.10 (Continued)



Finally, we examined the cellular events that normally occur during fusion in the chick to determine how overexpression of *Netrin-1* might be preventing fusion plate formation. Based on the similarity to the Bcl2 RCAS phenotype, we were particularly interested in the effects of Netrin-1 overexpression on cell death. After electroporation, tissue was harvested at E6.25, the stage when fusion normally occurs. In animals where the otic epithelium of the control unelectroporated ear was aligned but had not yet fused, the GFP-electroporated ear was identical to the control ear: as expected, the basement membrane was intact and cell death was evident in the otic epithelium where the fusion plate will form (Figure 2.11 A and B). Similarly, in animals where the otic epithelium of the control unelectroporated ear had already fused, the GFP-electroporated ear looked identical to the control ear: fusion had completed resulting in a mature canal (Figure 2.11 E and F). This is in line with the lack of a gross morphological defect in GFP electroporated ears at E7 as assessed by paintfilling (Figure 2.10 B). When Netrin-1-myc and GFP were co-electroporated, however, changes in the cellular events that underlie fusion were observed. In animals where the otic epithelium of the control unelectroporated ear was aligned and showed widespread apoptosis in the epithelium, the Netrin-1-myc electroporated ear lacked cell death in the epithelium (Figure 2.11 C vs. D). Furthermore, in animals where the otic epithelium of the control unelectroporated ear had already fused, the Netrin-1-myc electroporated ear lacked TUNEL positive cells in the epithelium, retained an intact basement membrane, and remained unfused (Figure 2.11 G vs. H). This suggests that overexpression of *Netrin-1* during canal morphogenesis in the chick interferes with the programmed cell death that is necessary for fusion to occur.

Figure 2.11: Overexpression of Netrin-1-myc interferes with fusion plate formation. CAG promoter driven Netrin-1-myc and GFP were coelectroporated into the right otic vesicle at HH stage 14-16. GFP was electroporated alone as a control. Immunostaining of tissue sections through the posterior canal at E6.25 are shown. A-H) laminin (red) marks the basement membrane and TUNEL (white) identifies apoptotic cells. B'-H') DAPI (white) identifies cell nuclei and GFP (green) identifies electroporated cells. Two stages were analyzed: when the otic epithelium of the control ear had not yet fused (A-D') and when the otic epithelium of the control ear had already fused (E-H'). Electroporation of GFP alone does not affect the fusion process. In animals where the otic epithelium of the control ear has not yet fused but apoptosis is evident in the center of the canal pouch epithelium (A), the GFP electroporated ear looks identical with cell death occurring in the center of the canal pouch epithelium (B). Similarly, in animals where the otic epithelium of the control ear has already fused (E), the GFP electroporated ear looks identical with the completion of fusion resulting in a mature canal (F). Conversely, co-electroporation of Netrin-1-myc and GFP interferes with fusion. In animals where the otic epithelium of the control ear has not yet fused but apoptosis is occurring in the center of the canal pouch epithelium (arrowhead C), the Netrin-1-myc electroporated ear lacks cell death in the center of the canal pouch epithelium (arrowhead D). Furthermore, in animals where the otic epithelium of the control ear has already fused (arrowhead G), the otic epithelium of the Netrin-1-myc electroporated ear still hasn't fused, lacks programmed cell death, and has an intact basement membrane (arrowhead H)

Figure 2.11 (Continued)



Since the loss of Netrin-1 in the mouse has been suggested to affect canal formation by decreasing proliferation in the mesenchyme surrounding the fusion plate (Salminen et al., 2000), we also examined proliferation in the mesenchyme after electroporation of Netrin-1-myc. ImageJ software was used to count PH3 positive cells in defined areas surrounding the fusion plate (see materials and methods). In animals where the otic epithelium of the control unelectroporated ear was aligned but had not yet fused, the Netrin-1-myc electroporated ear had a similar amount of proliferation in the mesenchyme, as assayed by PH3 staining (Figure 2.12 C and D). In animals where the otic epithelium of the control unelectroporated ear had already fused, however, the otic epithelium of the Netrin-1-myc electroporated ear failed to fuse despite a similar amount of proliferation in the surrounding mesenchyme (Figure 2.12 G and H, Table 2.2). Thus, it appears that Netrin-1 does not mediates its effects on fusion in the chick through regulation of mesenchymal proliferation as it does in mice (Salminen et al., 2000), although a more detailed analysis using EdU pulses to label diving cells will be necessary to make a definitive conclusion. Overall, we show that though Netrin-1 is involved in canal fusion in different species, it has divergent cellular effects between species.

Discussion

The results presented in this chapter highlight the importance of spatially regulated apoptosis during semicircular canal fusion in chicks. We showed that apoptosis precedes basement membrane breakdown during fusion, and that when apoptosis is inhibited by *Netrin-1* overexpression, basement membrane breakdown and

Figure 2.12: Electroporation mediated overexpression of Netrin-1-myc does not alter mesenchymal cell division as assayed by PH3 staining. CAG promoter driven Netrin-1-myc and GFP were coelectroporated into the right otic vesicle at HH stage 14-16. GFP was electroporated alone as a control. Immunostaining of tissue sections through the posterior canal at E6.25 are shown. DAPI (white) identifies cell nuclei, PH3 (red) identifies mitotic cells, and GFP (green) identifies electroporated cells. Two stages were analyzed: when the otic epithelium of the control ear had not yet fused (A-D) and when the otic epithelium of the control ear had already fused (E-H). Electroporation of GFP alone appears not affect mesenchymal cell proliferation. In animals where the otic epithelium of the control ear has not yet fused (A), the GFP electroporated ear displays similar mesenchymal PH3 staining (B). Similarly, in animals where the otic epithelium of the control ear has already fused (E), the GFP electroporated ear displays similar mesenchymal PH3 staining (F). Electroporation of Netrin-1-myc also appears not to affect mesenchymal cell proliferation as assayed by PH3 staining. In animals where the otic epithelium of the control ear has not yet fused (C), the Netrin-1-myc electroporated ear displays similar mesenchymal PH3 staining (D). Similarly, in animals where the otic epithelium of the control ear has already fused (G), the Netrin-1-myc electroporated ear displays similar mesenchymal PH3 staining (H).

Figure 2.12 (Continued)

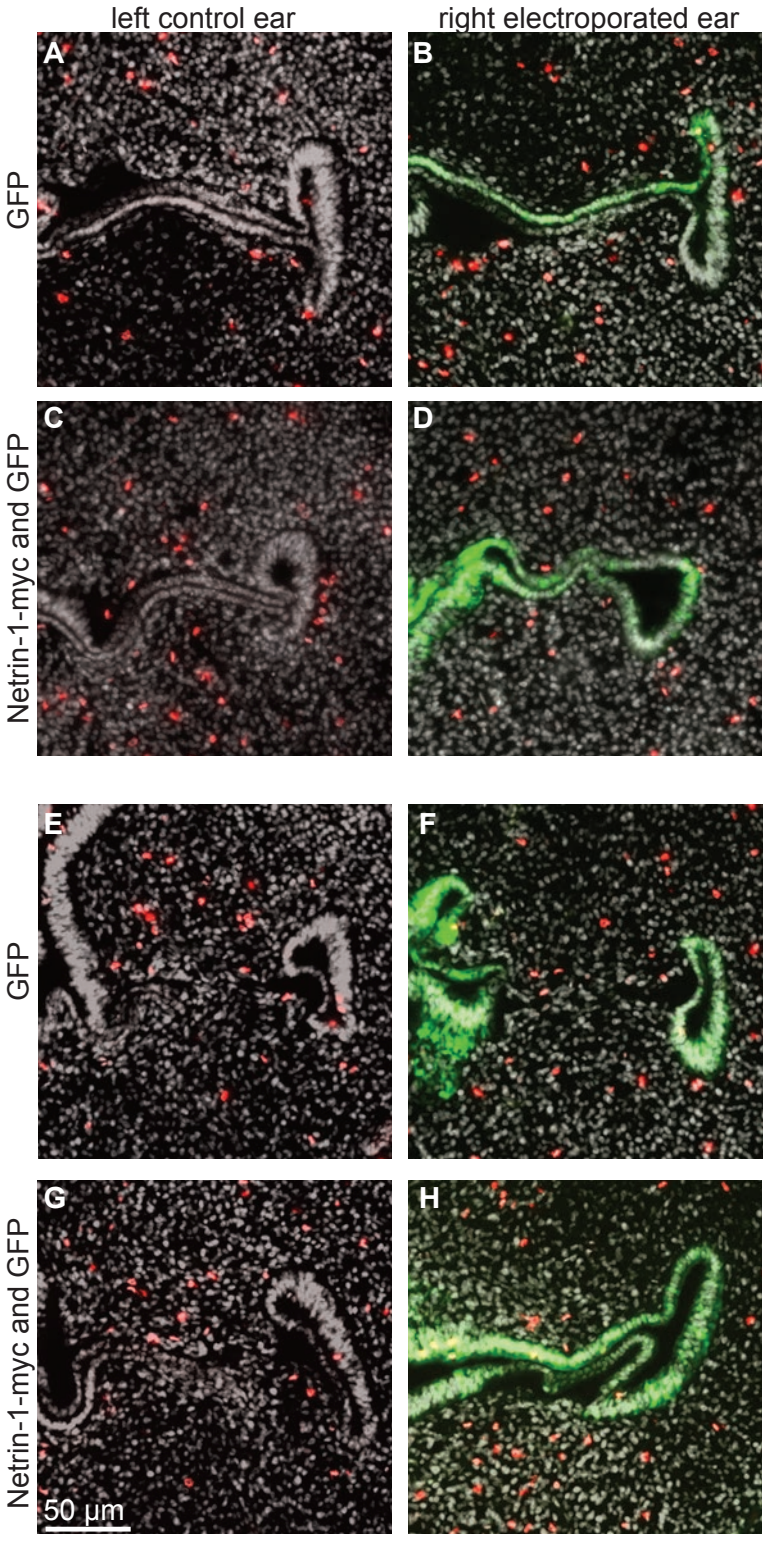


Table 2.2: Quantification of cell division in the mesenchyme after overexpression of Netrin-1-myc. PH3 immunostaining of tissue sections through the posterior canal before, during, and after fusion (E6-E6.25) was used to identify mitotic cells. As shown in Figure 2.3, regions of interest were defined manually using the DAPI channel, and ImageJ software was used to count the number of PH3 positive cells within the specified boxes. The cell counts were grouped into twelve groups: wild type aligned (box 1-3) and wild type fused (box 1-3) [shown in Table 2.1] and Netrin-1 overexpression (box 1-3) and GFP control (box 1-3) [shown in Table 2.2]. Each group was compared against every other group using a one-way analysis of variance. There was not a statistically significant difference in the mean number of PH3 positive cells across any of the groups as the p-value (0.386) was greater than 0.05.

Group	Sample size (number of tissue sections)	Mean number of PH3 positive cells per section
GFP control: box 1	10	2.5
GFP control: box 2	10	1.5
GFP control: box 3	10	2.5
Netrin-1-myc: box 1	9	2.22
Netrin-1-myc: box 2	9	2.22
Netrin-1-myc: box 3	9	2.56

fusion fail to proceed. These results strongly suggest that the function of cell death in canal morphogenesis is not merely to clear the fusion plate cells after fusion, as has generally been assumed. Rather, apoptosis may play additional, unexpected roles in the development of these exquisitely patterned structures.

Classically, apoptosis has been viewed as a developmental mechanism for removal of unnecessary tissue, as occurs during elimination of interdigital webbing (Garcia-Martinez et al., 1993). However, programmed cell death has many other functions during development (reviewed in Fuchs and Steller, 2011; Suzanne and Stellar, 2013). For instance, during palate formation, apoptosis plays a key role in the fusion of epithelial sheets or "shelves" at the midline of the oropharyngeal cavity to form a continuous palate (Mori et al., 1994; Cuervo et al., 2002). Detachment from the basement membrane can trigger apoptosis a process known as "anoikis." Anoikis is well-described phenomenon in both development and disease (reviewed in Grossman, 2002; Chiarugi and Giannoni, 2008). While both apoptosis and basement membrane breakdown occur during palatal fusion, detachment from the basement membrane does not trigger cell death in this context. On the contrary, apoptosis precedes basement membrane breakdown, and appears to trigger it. Blocking basement membrane breakdown does not prevent cell death while chemically triggering cell death causes basement membrane breakdown in the palatal shelves suggesting that activation of apoptotic pathways can trigger downstream effects on basement membrane integrity (Cuervo and Covarrubias, 2003). Cuervo and Covarrubias dub this type of cell death "cataptosis" and speculate that apoptosis-induced basement membrane breakdown

may be a conserved developmental mechanism, although no other similar examples had been described yet.

Our observation that apoptosis precedes basement membrane breakdown during semicircular canal fusion supports the idea that, as in palate development, cataptosis accompanies epithelial fusion events. Interestingly, both semicircular canal formation and palate formation involve the fusion of epithelial layers, suggesting that cataptosis may be conserved during epithelial fusion events. However, it remains to be tested if apoptosis during canal fusion triggers basement membrane breakdown, as in palate development. While it will be more difficult to trigger apoptosis or block basement membrane breakdown *in ovo*, similar pharmacological manipulations as those used by Cuervo and Covarrubias can be used to test this.

Overexpression of Netrin-1-myc in the chick inner ear by either RCAS virus or electroporation prevents apoptosis at the fusion plate and interferes with subsequent canal fusion. The fact that apoptosis begins at the fusion plate before fusion (Lang, et al, 2000; Figure 2.2 A'') suggests that apoptosis does not function solely to clear fusion plate cells after fusion, but instead plays a role in initiating fusion plate formation. Aside from potentially initiating basement membrane breakdown, which has been hypothesized to allow for diffusion of secreted signaling molecules (Abraira et al., 2008), apoptosis at the fusion plate could also function early to disrupt epithelial cell-cell interactions and allow for the intercalation of the opposing epithelial layers.

Netrin-1 has previously been shown to promote cell survival by binding to a subset of Netrin receptors. Both DCC and Unc5 can act as dependence receptors, which trigger cell death in the absence of ligand. For example, expression of DCC in

cells that do not normally express this receptor triggers apoptosis, while the addition of Netrin-1 prevents cell death (Mehlen et al., 1998). *In vivo*, DCC and Unc5 both exhibit loss of heterozygosity and downregulation in a variety of cancers, suggesting that these receptors may normally prevent cell survival (reviewed in Mehlen et al., 2011). Conversely, increased *Netrin-1* expression is associated with many types of cancer, including breast cancer (Fitament et al., 2008), lung cancer (Delloye-Bourgeois et al., 2009a), pancreatic cancer (Link et al., 2007), and neuroblastoma (Delloye-Bourgeois et al., 2009b).

Aside from a role in cancer, there is preliminary evidence in *Drosophila* that Netrins can act *in vivo* as a trophic factor. *NetAB* double knockout animals exhibit the expected defects in commissural axon midline guidance (Newquist et al., 2013), similar to the *Netrin-1* phenotype in mice (Serafini et al., 1996), as well as increased cell death. As expected, pan-neuronal expression of *NetA* in double mutants caused more severe defects in midline guidance due to the presence of a non-directional Netrin signal, in line with the long-held belief that NetA is a chemotropic guidance cue (Harris et al., 1996). Pan-neuronal expression of *NetB* in these double mutants reduced apoptosis and unexpectedly rescued the midline guidance defects, even though the Netrin signal was not directional. This suggested that NetB acts mainly as a trophic factor to promote survival. Indeed, simply blocking apoptosis was able to rescue the midline guidance defects in *NetAB* double mutants, providing *in vivo* evidence that the survival and guidance activities of Netrin are functionally related (Newquist et al., 2013) and that in species with multiple Netrin isoforms, different Netrins can perform different functions.

Netrins are expressed early in the otic epithelium and are known to control many important cellular events by signaling through numerous receptors; therefore, it is likely that Netrins play multiple roles during inner ear morphogenesis. Since Netrins can promote survival, it was surprising that Netrin-1 and -2 are normally expressed within the canal fusion plate, exactly where apoptosis occurs. We propose a model in which the restriction of fusion to the center of the canal pouch is determined by the relative expression levels of Netrin-1 and its dependence receptors (Figure 2.13). We hypothesize that both the fusing and non-fusing domains of the canal pouch epithelium express dependence receptors, but that they are expressed at higher levels in the fusion plate. In wildtype animals, only a subset of cells produce Netrin-1. We propose that this amount of Netrin-1 is sufficient to prevent dependence receptor mediated apoptosis in the non-fusing canal rim. In the fusing epithelium, there is insufficient Netrin-1 to bind all dependence receptors; unbound receptors lead to apoptosis and initiation of fusion. When the Netrin-1 expression domain is expanded, on the other hand, more Netrin-1 is produced. We propose that these higher Netrin-1 levels are sufficient to prevent unbound dependence receptor mediated apoptosis and initiation of fusion in the center of the canal pouch. This accounts for the lack of apoptosis and fusion observed in both our overexpression manipulations (Figures 2.7, 2.11, and 2.12).

Netrin-1 is expressed in the center of the canal pouch long before fusion and its expression remains in the rim of the semi-circular canal long after fusion. This persistent expression suggests that it likely plays important roles in this

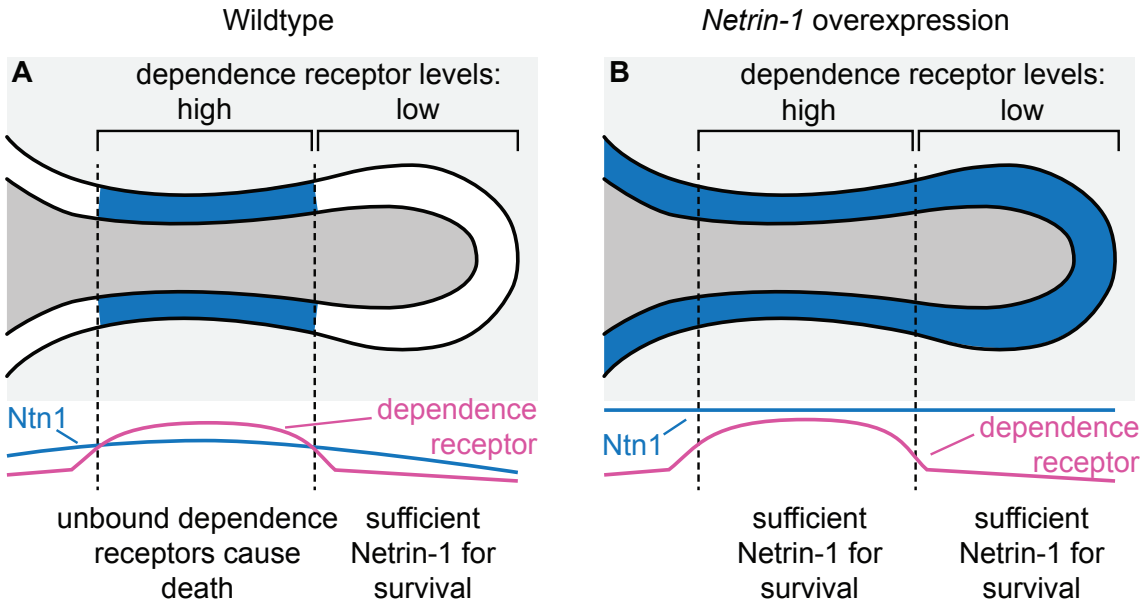


Figure 2.13: Proposed role for Netrin-1 as a trophic factor during canal formation. One model that could account for our data is that restricted fusion in the center of the canal pouch is determined by the relative expression levels of Netrin-1 (blue) and its dependence receptors (pink). We hypothesize that both the fusing and non-fusing domains of the canal pouch epithelium express dependence receptors, but that they are expressed at higher levels in the fusion plate. In wildtype animals (A), only a subset of cells produce Netrin-1. We propose that this amount of Netrin-1 is sufficient to prevent dependence receptor mediated apoptosis in the non-fusing canal rim. In the fusing epithelium, there is insufficient Netrin-1 to bind all dependence receptors and unbound receptors lead to apoptosis and initiation of fusion. When the Netrin-1 expression domain is expanded (B), more Netrin-1 is produced. We propose that these higher Netrin-1 levels are sufficient to prevent unbound dependence receptor mediated apoptosis and initiation of fusion in the center of the canal pouch.

tissue beyond initiating fusion. In the chick, however, initiation of fusion is believed to require apoptosis in the center of the canal pouch epithelium. Two possible mechanisms to generate apoptosis in this Netrin-1 expressing region include downregulation of Netrin-1 during fusion or upregulation of dependence receptors. Since Netrin-1 levels appear to remain constant, we propose that apoptosis is generated in this tissue by upregulation of dependence receptors to a level exceeding the amount of ligand present.

Determining the spatiotemporal patterns of Netrin receptor expression during inner ear development may provide important insights into the role of Netrin-1 in the otic epithelium across development. It will also be important to determine if loss of Netrin function in chicks promotes cell death. To this end, we have begun preliminary work overexpressing the ectodomain of DCC, which binds Netrin and acts as a dominant negative molecule (Stein et al., 2001; Fitament et al., 2008).

Overall, our results suggest that Netrin-1 plays divergent roles during canal morphogenesis in the chick and mouse. While overexpression of Netrin-1-myc in the chick prevents fusion, expanded *Netrin-1* expression in the lateral canal of *Lrig3* mutant mice causes ectopic fusion and canal truncation (Abraira et al., 2008). In fact, loss of *Netrin-1* in mice interferes with fusion, the same phenotype we observe with overexpression of *Netrin-1* in chicks, which strongly suggests that Netrin-1 has different functions across species during fusion. One obvious difference is that cell death does not appear to be involved in canal fusion in the mouse (Martin and Swanson, 1993; Nishikori 1999 et al.).

Additionally, in other tissues, Netrin-1 function in mammals appears to be represented by the combined action of Netrin-1 and Netrin-2 in chicks. Thus, it is possible that Netrin-2 has a different role than Netrin-1 in chick semicircular canal formation, similar to the different roles of NetA and NetB in midline guidance in the fly (Newquist et al., 2013). For instance, while we show that Netrin-1 regulates cell death at the fusion plate, Netrin-2 may regulate basement membrane breakdown at the fusion plate, similar to the role of Netrin-1 in mice (Salminen et al., 2000; Abraira et al., 2008). In the future, it will be interesting to determine if overexpression of *Netrin-2* in the developing chick inner ear has the same cellular effects as overexpression of *Netrin-1*. Finally, *Netrin-1* expansion in *Lrig3* mice is limited to the lateral canal epithelium and results from changes in a repressive transcriptional feedback loop. While removing one copy of *Netrin-1* in the *Lrig3* mutant background rescues the canal truncation defect, a result which strongly suggests that the phenotype is a result of increased *Netrin-1* expression (Abraira et al., 2008), it is possible that other factors downstream of *Lrig3* contribute to canal formation. To more directly compare the role of Netrin-1 in the chick and mouse, we have directly induced ectopic expression of *Netrin-1* throughout the entire otic epithelium in mice; this work is described in the next chapter of this dissertation.

Our results demonstrate a novel function for Netrin-1 as a trophic factor during canal morphogenesis in chicks, and indicate that despite being highly evolutionarily conserved, Netrins perform the same functions through divergent mechanisms across different organisms.

Chapter 3: Creation of a *Netrin-1* conditional expression allele in mouse

Allison M. Nishitani and Lisa V. Goodrich.

A.M. Nishitani created the *Netrin-1* conditional expression mouse line and performed analysis on inner ear morphology in these mice. Blastocyst injections were performed by the Boston Children's Hospital transgenic core. The *Crect* line was provided by Trevor Williams, University of Colorado Denver.

Introduction

Overexpression of *Netrin-1* in the developing chick inner ear interferes with fusion; this is the opposite of the excessive fusion and canal truncation observed with ectopic *Netrin-1* expression in mice (Arbraira et al., 2008). In fact, at the gross level, overexpression of *Netrin-1* in the chick inner ear resembles the failure of fusion reported with loss of *Netrin-1* in mice (Salminen et al., 2000). We propose in Chapter 2 that differences in the main cellular effect of Netrin-1 during semicircular canal formation in chicks and mice underlie the different morphological phenotypes observed.

In Chapter 2 we show that Netrin-1 acts as a survival factor in the developing chick inner ear and that overexpression of *Netrin-1* prevents the apoptosis required for fusion in chicks (Fekete et al., 1997). On the other hand, it seems that the main cellular effect of Netrin-1 during fusion in mice is basement membrane regulation. In mice, loss of *Netrin-1* prevents basement membrane breakdown at the fusion plate (Salminen et al, 2000), but it remains unclear whether ectopic *Netrin-1* is sufficient to cause basement membrane breakdown during fusion in mice. As described in the general introduction, mice that have expanded *Netrin-1* expression in the lateral canal pouch epithelium as a result of *Lrig3* loss, have ectopic basement membrane breakdown and fusion, resulting in lateral canal truncation (Abraira et al., 2008). While loss of one copy of *Netrin-1* was sufficient to rescue canal truncation in *Lrig3* mutants, it is possible that other factors downstream of *Lrig3* contribute to canal formation (Abraira et al., 2008), and that the different morphological phenotypes observed with increased *Netrin-1* in chicks and mice is a result of factors downstream of *Lrig3*.

Arguing against this possibility, here we show that direct expression of *Netrin-1* throughout the otic epithelium is sufficient to cause canal truncation in mice, providing evidence that the main cellular effect of Netrin-1 during fusion in mice is the regulation of basement membrane breakdown during fusion. Further, because we ectopically expressed chick *Netrin-1* in the mouse, we bolster our argument that Netrin-1 has divergent functions during canal formation, as overexpression of the same protein in two species caused opposite morphological phenotypes.

Materials and methods

Mice

As described in this chapter, the *Netrin-1* conditional expression line ($Ntn1^{CE}$) contains a Cre-dependent CAG promoter driven Netrin-1-myc – IRES – tdTomato – pA cassette inserted into the *Rosa26* locus (Figure 3.1). PCR primer pairs that detect the presence or absence of the tdTomato insertion in the *Rosa26* locus allow for the detection of homozygous $Ntn1^{CE/CE}$ animals.

tdTomato forward (CTGTTCCTGTACGGCATGG) and

tdTomato reverse (GGCATTAAAGCAGCGTATCC);

control forward (AAGGGAGCTGCAGTGGAGTA) and

control reverse (CCGAAAATCTGTGGGAAGTC)

ES cell screening

ES cell clones were screened using PCR reactions that amplify the 5' and 3' homology regions of the *Rosa26* locus:

5' arm forward (CGCCTAAAGAAGAGGCTGTG) and

5' arm reverse (GGGCGTACTTGGCATATGAT);

3' arm forward (GCCTCGACTGTGCCTTCTAG) and

3' arm reverse (CCATTCTCAGTGGCTCAACA).

These primers generate 1472 bp and 4886 bp products, respectively, if recombination has occurred correctly. Additionally, to verify Cre-mediated induction of Netrin-1 expression, ES clones were transfected with Cre-GFP, GFP, or no DNA using Lipofectamine (Life Technologies) and cell lysate was used for Western blot analysis, as described below.

Paintfilling

E14.5 heads were fixed overnight in Bodian fix at 4°C, washed for 10 minutes in 100% ethanol, and dehydrated overnight in 100% ethanol at room temperature. Samples were then rinsed briefly in methyl salicylate and cleared overnight in methyl salicylate at room temperature. Heads were hemisected along the midline and their cochleae were injected from the medial side with White-out (BIC) diluted to 0.025% with methyl salicylate using a pulled glass pipette and a Hamilton syringe.

Western blot

E11.5 forebrains were lysed using an electronic homogenizer and 200 µl of the following lysis buffer: 50 mM Tris pH7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycolate, 0.1% SDS, 1x Pefabloc SC PLUS protease inhibitor (Roche). ES cells were lysed using the same lysis buffer. The following primary antibodies were used: Netrin-1 (1:500, R&D MAB1109) and myc (1:500, Santa Cruz SC-56633).

Immunohistochemistry

Embryonic tissue was fixed overnight at 4°C in 4% PFA/PBS and washed 3 times for 10 minutes in PBS. Tissue was then put through a sucrose series (10% sucrose/PBS, 20% sucrose/PBS, 30% sucrose/PBS) and transitioned into Neg-50 embedding media (Richard-Allen Scientific) with incubation in each solution overnight at 4°C. Tissue was embedded in Neg-50 by freezing with a slurry of dry ice and isopentane. 12-14 µm cryosections were counterstained with DAPI (1:10,000) and mounted using Vectashield mounting media (Vector labs).

Results

Creation of $Ntn1^{CE}$ mice

The coding sequence of chicken Netrin-1 with a C-terminal myc tag was cloned into the AscI site of a Rosa26 targeting vector (Ctd), which contains a floxed stop and an IRES - tdTomato under the control of a strong CAG promoter (Yu et al., 2013) (Figure 3.1 A). Netrin-1 with a C-terminal myc tag has equivalent outgrowth activity to untagged Netrin-1 (Serafini et al., 1994) and is assumed to be biologically active. We

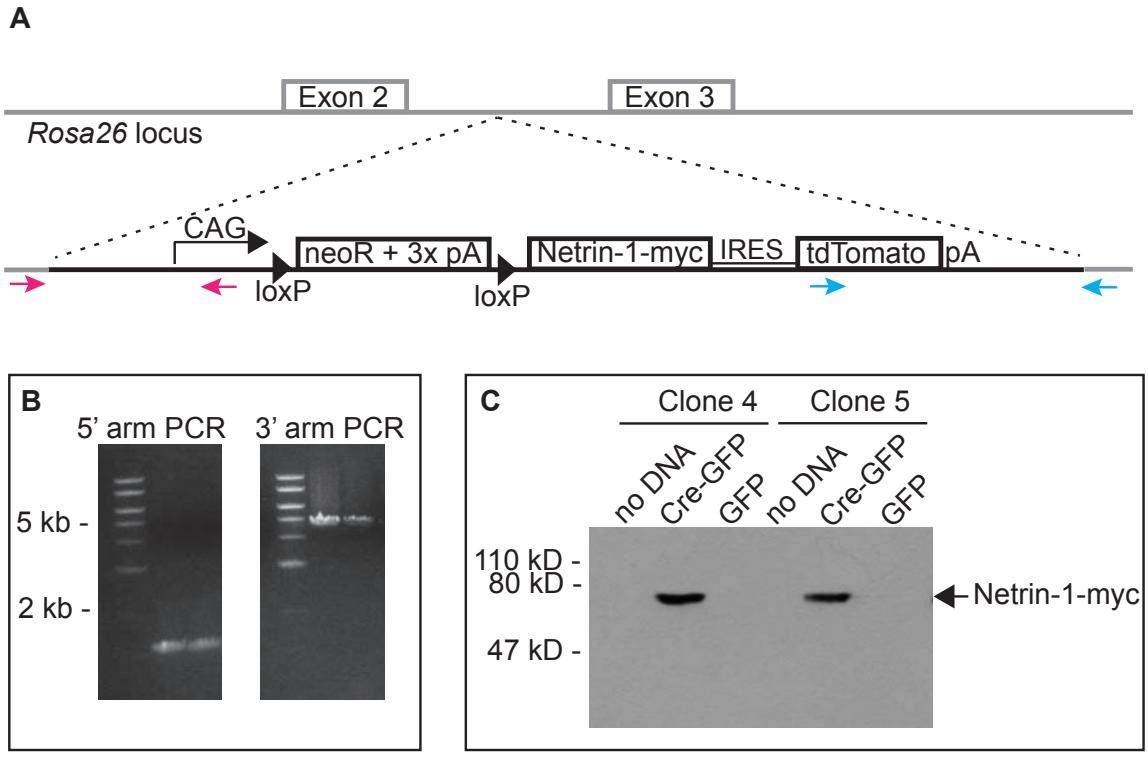


Figure 3.1: Design of the *Ntn1*^{CE} allele. A) The *Ntn1*^{CE} allele contains a Cre dependent CAG promoter driven Netrin-1-myc – ires – tdTomato - pA cassette inserted into the *Rosa26* locus. B) ES cell clones were screened for 5' and 3' recombination by PCR using primers that spanned the junctions between the endogenous *Rosa26* locus and the homologous regions of the targeting cassette (5' recombination primers shown in pink generate a 1472 bp product, 3' recombination primers shown in blue generate a 4886 bp product). C) After PCR screening, two ES cell clones were selected for additional validation. ES cells were transfected with Cre-GFP, GFP, or no DNA and cell lysate was blotted for myc to detect the production of Netrin-1-myc protein. Both clones showed production of Netrin-1-myc protein only upon introduction of Cre.

included the myc tag due to the lack of effective antibodies that detect Netrin-1 by immunohistochemistry.

The targeting construct was linearized using SgfI, electroporated into J1 ES cells (derived from 129S4/SvJae strain), and selected under G418 for 1 week. 48 ES cell clones were screened for correct recombination by PCR using primers to amplify the 5′ and 3′ homology regions of the *ROSA26* locus (Figure 3.1 B). 31/48 clones were positive for 5' and 3' homologous recombination. Four clones (#2-5) were selected for karyotyping. Less than 50% of cells in clones 2 and 3 had the correct number of chromosomes, while 95% of cells in clones 4 and 5 had the correct number of chromosomes. To verify Cre-mediated induction of *Netrin-1* expression, ES clones 4 and 5 were transfected with Cre-GFP, GFP, or no DNA. As expected, myc-tagged Netrin-1 was present in cell lysate only upon addition of Cre (Figure 3.1 C). Clone 4 was used for blastocyst injection. 12 chimeric males were born and the presence of the $Ntn1^{CE}$ allele in their progeny was detected using PCR primers that amplify tdTomato. Germline transmission occurred in 3 chimeric males, yielding $Ntn1^{CE/+}$ mice. $Ntn1^{CE/CE}$ homozygous mice are viable and fertile.

The effects of ectopic Netrin-1 expression on canal morphogenesis

$Ntn1^{CE/+}$ mice were crossed with two different Cre drivers to induce expression in the otic epithelium. Expression of myc-tagged Netrin-1 in tissue sections was undetectable by immunostaining, likely due to the presence of only one copy of the tag. However, since the $Ntn1^{CE}$ allele contains an IRES – tdTomato element, recombination could be visualized by presence of tdTomato.

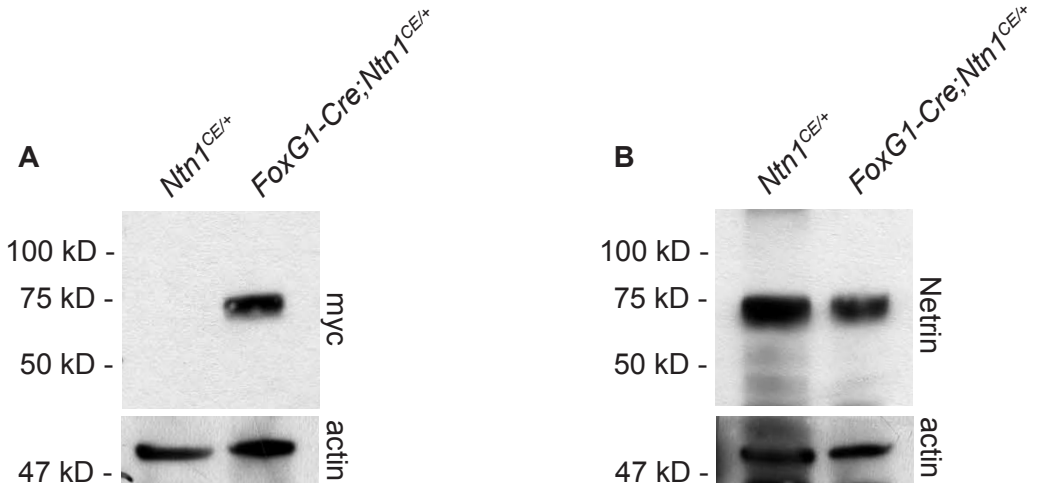


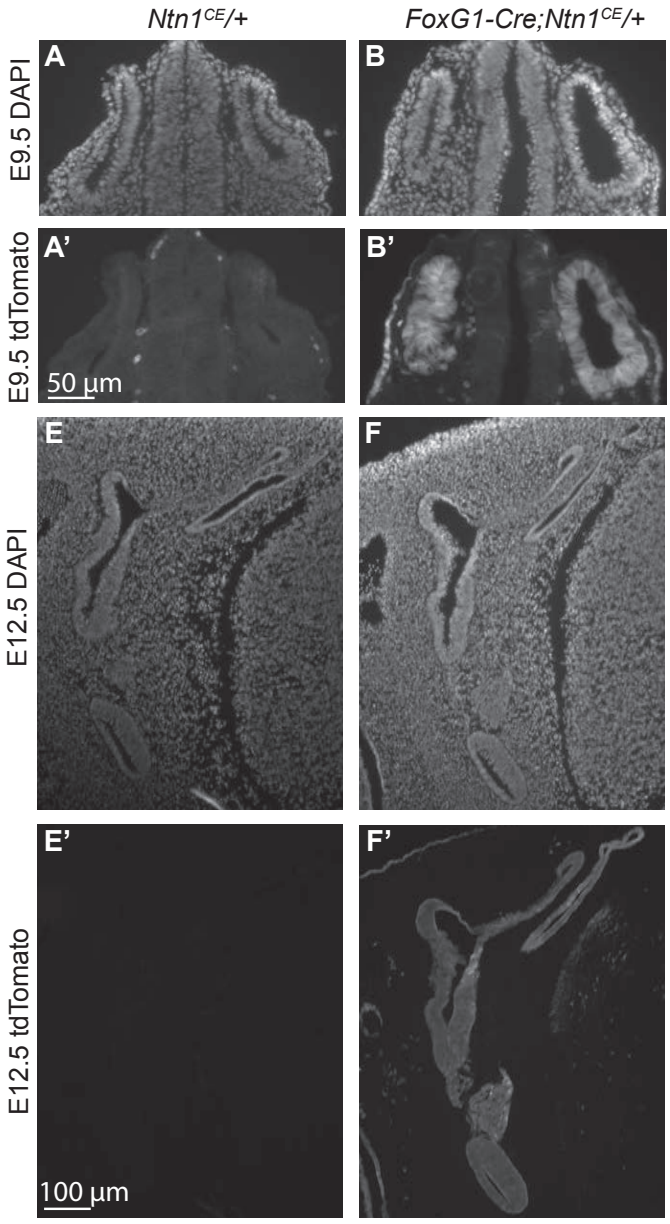
Figure 3.2: Cre mediated induction of Netrin-1-myc protein expression. Western blot analysis of E12.5 mouse forebrain lysate from *Ntn1*$^{CE/+}$ and *FoxG1-Cre;Ntn1*$^{CE/+}$ animals. A) Blotting with an antibody against myc shows the production of Netrin-1-myc protein only in the presence of Cre. B) Blotting with an antibody against Netrin shows that the overall levels of Netrin-1 protein are similar in *Ntn1*$^{CE/+}$ and *FoxG1-Cre;Ntn1*$^{CE/+}$ lysates, suggesting a down regulation of endogenous Netrin-1 in the presence of exogenous Netrin-1-myc. Actin is shown as a loading control.

FoxG1-Cre drives early recombination in the otic epithelium (~E8.75) (Hebert and McConnel, 2000). At E9.5, almost every cell in the otic epithelium of *FoxG1-Cre;Ntn1*$^{CE/+}$ mice expressed tdTomato (Figure 3.3 B'), and by E12.5, the entire otic epithelium was tdTomato positive (Figure 3.3 F'). FoxG1-Cre is also active in the developing forebrain. To verify the production of myc-tagged Netrin-1 protein, lysate from E11.5 forebrains was used for Western blot analysis; the forebrain was chosen due to the relative abundance of this tissue. While production of myc-tagged exogenous Netrin-1 was evident in *FoxG1-Cre;Ntn1*$^{CE/+}$ lysate (Figure 3.2 A), we were unable to detect the substantial increase in total Netrin-1 protein that would be expected when ectopic *Netrin-1* expression is driven using a strong CAG promoter (Figure 3.2 B). This may suggest that Netrin-1 is autoregulated in the brain.

To determine if ectopic *Netrin-1* is sufficient to cause canal truncation we paintfilled ears from E14.5 *FoxG1-Cre;Ntn1*$^{CE/+}$ mice to assess overall morphology, paying particular attention to the formation of the canals. *FoxG1-Cre;Ntn1*$^{CE/+}$ ears displayed defects in formation of all three canals (Figure 3.4). Lateral canal defects included both thinning (Figure 3.4 C, n=1) and complete truncation (Figure 3.4 D, n=3). Anterior canal truncation was observed in a small number of *FoxG1-Cre;Ntn1*$^{CE/+}$ ears (Figure 3.4 E, n=2) and one posterior canal defect was observed (Figure 3.4 F, n=1). Despite the early and broad expression of the Netrin-1 transgene, the majority of *FoxG1-Cre;Ntn1*$^{CE/+}$ ears were unaffected and exhibited normal canal morphology (Figure 3.4 B, n=42/49). Previous work showed that increased Netrin-1 in the lateral canal due to loss of *Lrig3* causes a lateral canal truncation (Abraira et al., 2008). Our experiments show that increased levels of Netrin-1 in the entire otic epithelium can

Figure 3.3: Induction of Netrin-1-myc expression using FoxG1-Cre. FoxG1-Cre turns on early in the developing otic vesicle ~E8.75. While detection of the Netrin-1-myc fusion protein was not possible by immunostaining, the presence of an IRES tdTomato in the allele allowed for the use of tdTomato staining to track recombination of the $Ntn1^{CE}$ allele (A'-B' and E'-F'). Expression was checked early during the otic vesicle stage at E9.5 (A-B') and later just before fusion at E12.5 (E-F'). At E9.5, tdTomato was evident in almost every cell of the otic vesicle in *FoxG1-Cre;*$Ntn1^{CE/+}$ animals (B') but not in $Ntn1^{CE/+}$ animals lacking Cre (A'). At E12.5, tdTomato was evident in the entire otic epithelium in *FoxG1-Cre;*$Ntn1^{CE/+}$ animals (F') but not in $Ntn1^{CE/+}$ animals lacking Cre (E').

Figure 3.3 (Continued)



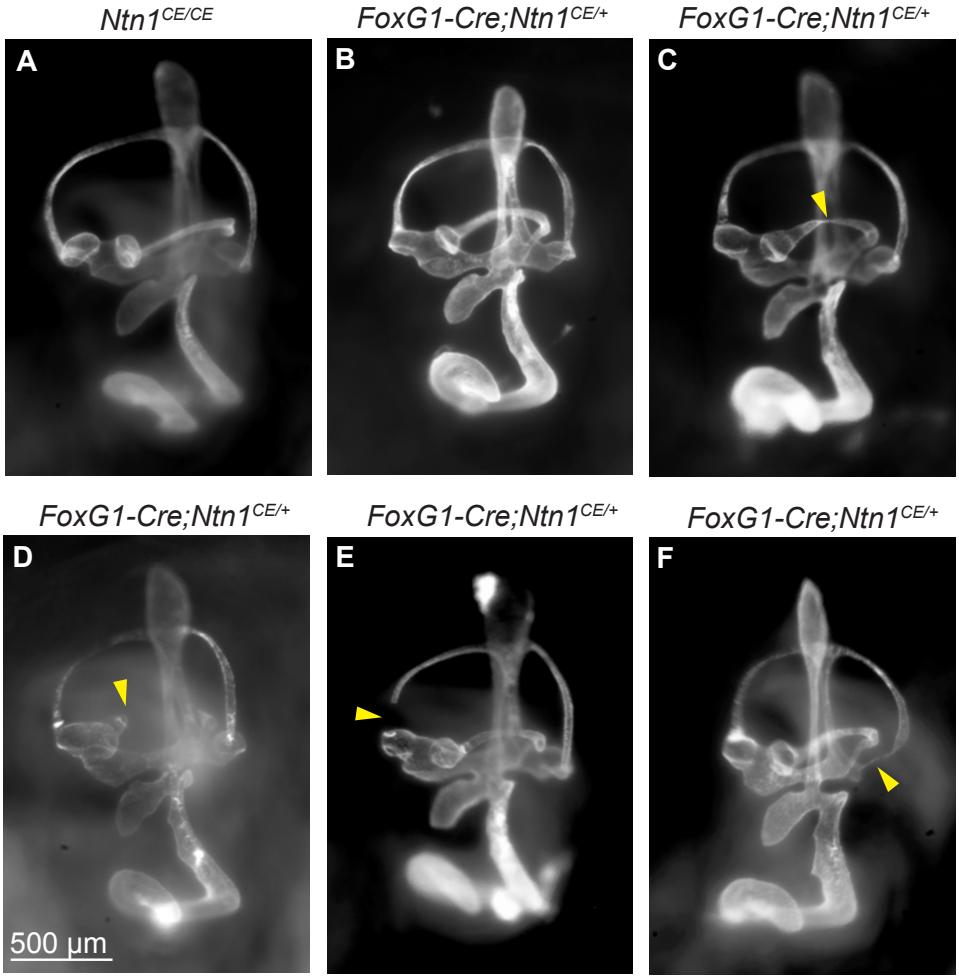


Figure 3.4: Targeted expression of Netrin-1-myc throughout the otic epithelium using FoxG1-Cre can disrupt canal formation. Paintfilling of the mouse inner ear at E14.5. A) *Ntn1*$^{CE/CE}$ homozygotes have normal canal morphology (n=10). B) A large fraction of *FoxG1-Cre;Ntn1*$^{CE/+}$ ears are unaffected (n=42/49). C-D) The lateral canal was affected infrequently in *FoxG1-Cre;Ntn1*$^{CE/+}$ ears, displaying both thinning (arrowhead C, n=1/49) and complete truncation (arrowhead D, n=3/49). E-F) Rare anterior truncations (arrowhead E, n=2/49) and posterior canal defects (arrowhead F, n=1/49) were also observed.

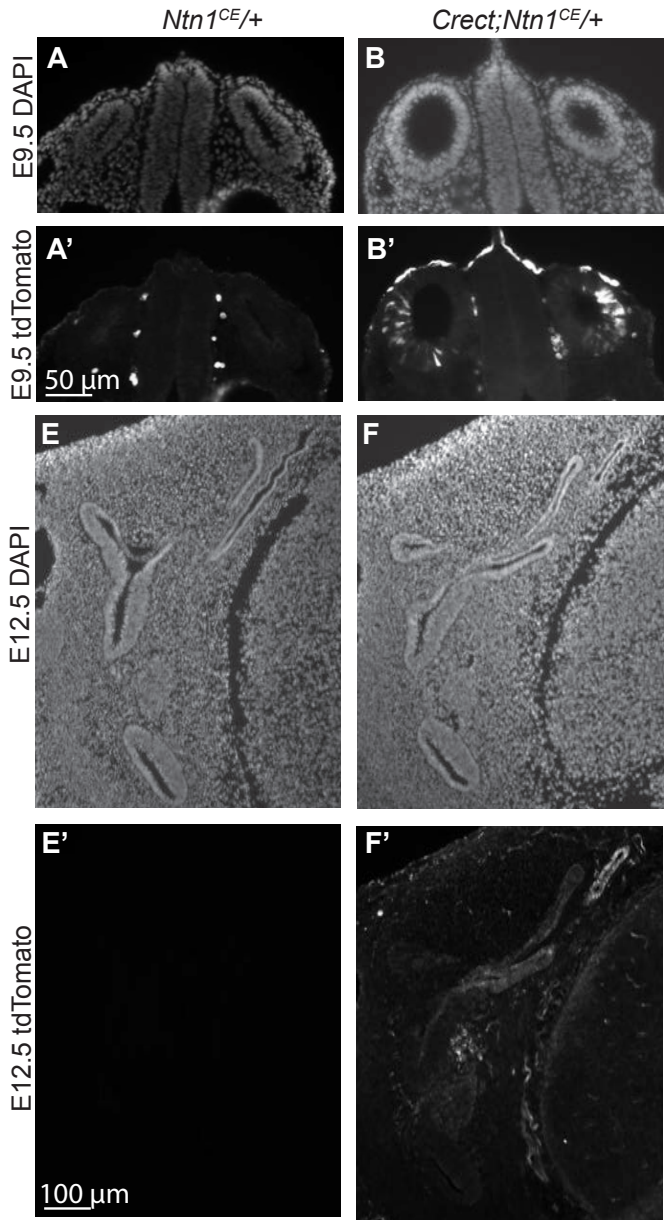
cause similar defects in formation of all three semicircular canals. While *FoxG1-Cre;Ntn1*$^{CE/+}$ mice usually do not survive to postnatal ages, a few mice of this genotype survived. 2/3 adult *FoxG1-Cre;Ntn1*$^{CE/+}$ mice exhibited circling and head bobbing behaviors characteristic of vestibular defects.

Since the lateral canal truncation observed in *Lrig3* mutants was 100% penetrant on an inbred C57BL/6 background (Abraira et al., 2008), the relatively low 14% penetrance of canal defects in *FoxG1-Cre;Ntn1*$^{CE/+}$ ear was unexpected. *In vitro*, excessive Netrin-1 has inhibitory effects on axon outgrowth (Serafini et al., 1994; Metin et al., 1997). We wondered, therefore, whether FoxG1-Cre was driving ectopic Netrin-1 expression in an overly broad area of the otic epithelium and inhibiting Netrin-1 function. An alternate Cre driver, *Crect*, uses a head ectoderm-specific enhancer of the AP2α promoter (Forni et al., 2011; Harlow et al., 2011). This Cre line was designed to drive early recombination (E8.5) in all cranial placodes and head ectoderm, but in our hands, Crect drives very sparse recombination in the otic epithelium at E9.5 (Figure 3.5 B'). Even at E12.5, recombination in the otic epithelium remained sparse and uneven (Figure 3.5 F'). Thus, we were able to use *Crect/+;Ntn1*$^{CE/+}$ mice to test the effects of salt-and-pepper ectopic expression of Netrin-1 in the otic epithelium.

Ears from E14.5 *Crect;Ntn1*$^{CE/+}$ mice were paintfilled to assess overall morphology, with particular attention paid to the formation of the canals (Figure 3.6). While canal truncations were observed in 14% of *FoxG1-Cre;Ntn1*$^{CE/+}$ ears (n=7/49 affected), 26% of *Crect;Ntn1*$^{CE/+}$ ears displayed canal truncations (n=9/34 affected), suggesting that ectopic salt-and-pepper expression of *Netrin-1* gives a more penetrant canal truncation phenotype. Interestingly, with the use of Crect, only the formation of the

Figure 3.5: Induction of Netrin-1-myc expression using Crect. Crect drives sparse recombination in the otic epithelium. While detection of the Netrin-1-myc fusion protein was not possible by immunostaining, the presence of an IRES tdTomato in the allele allowed for the use of tdTomato staining to track recombination of the $Ntn1^{CE}$allele (A'-B' and E'-F'). Expression was checked early during the otic vesicle stage at E9.5 (A-B') and later just before fusion at E12.5 (E-F'). At E9.5, tdTomato was evident in just a few cells of the otic epithelium in *Crect;*$Ntn1^{CE/+}$ animals (B') but not in $Ntn1^{CE/+}$ animals lacking Cre (A'). At E12.5, tdTomato was evident at varying levels in the otic epithelium in *Crect;*$Ntn1^{CE/+}$ animals (F') but not in $Ntn1^{CE/+}$ animals lacking Cre (E').

Figure 3.5 (Continued)



lateral canal was affected. Both thinning of the lateral canal (Figure 3.6 C, n=2) and lateral canal truncation (Figure 3.6 D, n=9) were observed in *Crect;Ntn1*$^{CE/+}$ ears.

Discussion

While Netrins are highly conserved and mediate many important cellular events during development and disease, we lack a complete understanding of core Netrin-1 function *in vivo.* We sought to better define the main cellular effect of Netrin-1 *in vivo* during canal morphogenesis in chicks and mice. While there is direct evidence that the loss of *Netrin-1* in mice prevents basement membrane breakdown at the fusion plate (Salminen et al, 2000), the effect of ectopic *Netrin-1* on the basement membrane is less clear. As described earlier, mice that have expanded *Netrin-1* in the lateral canal pouch epithelium as a result of *Lrig3* loss, showed lateral canal truncation (Abraira et al., 2008). We created the *Netrin-1* conditional expression mouse (*Ntn1*^{CE}) to directly induce ectopic *Netrin-1* expression *in vivo*, allowing us to study the effect of *Netrin-1* overexpression on the basement membrane.

We began our analysis by directly inducing ectopic expression of *Netrin-1* throughout the entire otic epithelium in order to determine whether ectopic *Netrin-1* expression is sufficient to cause truncation of the lateral canal as in *Lrig3* mutants, and of the anterior and posterior canals, which are unaffected in *Lrig3* mutants (Abraira et al., 2008). While the lateral canal was affected more frequently in *FoxG1-Cre;Ntn1*$^{CE/+}$ ears, defects in both the anterior and posterior canals were observed, indicating that the ectopic *Netrin-1* is sufficient to cause canal truncation in all three canals. This is in line

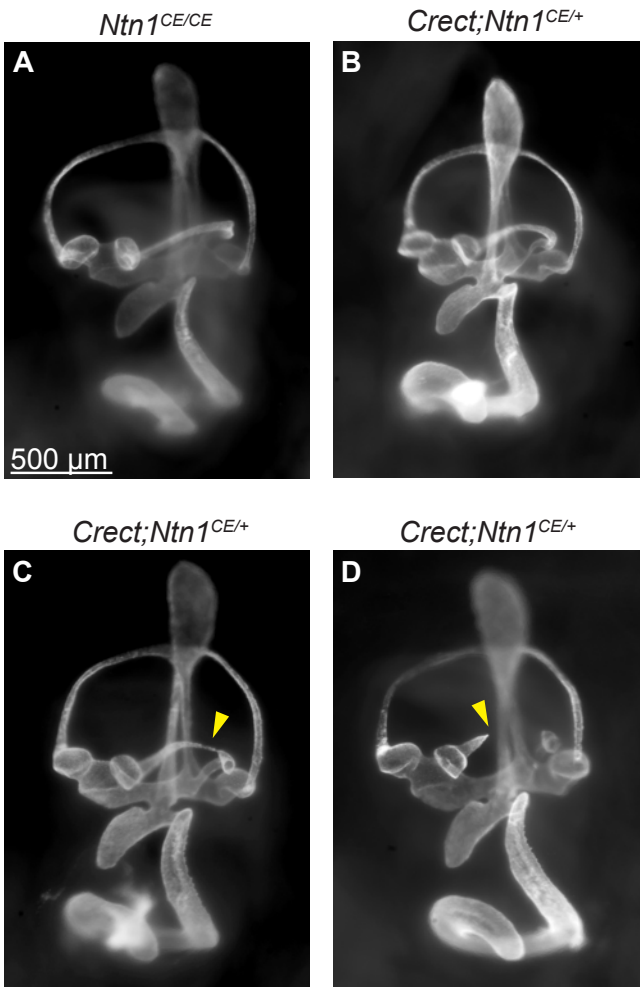


Figure 3.6: Targeted expression of Netrin-1-myc using Crect can disrupt canal formation. Paintfilling of the mouse inner ear at E14.5. A) *Ntn1*$^{CE/CE}$ homozygotes develop normal canal morphology (n=10). B) Many *Crect;Ntn1*$^{CE/+}$ ears are normal (n=23/34). C-D) Only the lateral canal was affected in *Crect;Ntn1*$^{CE/+}$ ears, with both thinning (arrowhead C, n=2/34) and complete truncation (arrowhead D, n=9/34) observed.

with the fact that loss of Netrin-1 causes fusion defects in all three canals (Salminen et al., 2000).

Our experiments suggest that the effects of Netrin-1 on the basement membrane may also be concentration dependent. We were prompted to explore the effect of varying levels of ectopic *Netrin-1* after finding a relatively low penetrance of the *FoxG1-Cre;Ntn1*$^{CE/+}$ canal truncation phenotype (14%) compared to 100% penetrance of the lateral canal truncation observed in *Lrig3* mutants (Abraira et al., 2008). *In* vitro assays demonstrate that Netrin-1 function is concentration dependent, with high levels of Netrin-1 inhibiting its activity. Therefore, we used the *Crect* line to drive sparse recombination in the otic epithelium to test the hypothesis that the weak FoxG1-Cre phenotype was caused by inhibitory effects due to overly broad ectopic *Netrin-1* expression. In line with this, the penetrance of *Crect;Ntn1*$^{CE/+}$ canal defect was increased (26%, n=9/34 affected) compared to *FoxG1-Cre;Ntn1*$^{CE/+}$ ears (14%, n=7/49 affected). Importantly, the fact that canal truncation was observed in both *FoxG1-Cre;Ntn1*$^{CE/+}$ and *Crect;Ntn1*$^{CE/+}$ ears despite the varying patterns of recombination suggests that different levels of Netrin-1 during canal formation do not exert different cellular effects.

It is difficult to differentiate between the effects of timing, location, and levels of recombination between FoxG1-Cre and Crect. The only factor that can be assessed relatively easily is the amount of ectopic Netrin-1 produced using the different Cre lines. To determine this, we are collecting otic vesicles from both *FoxG1-Cre;Ntn1*$^{CE/+}$ and *Crect;Ntn1*$^{CE/+}$ animals to perform Western blot analysis to compare exogenous Netrin-1 levels in the otic epithelium after Cre-mediated recombination. We expect *Crect;Ntn1*$^{CE/+}$

otic vesicles to express less Netrin-1-myc than *FoxG1-Cre;Ntn1*$^{CE/+}$ samples based on the much sparser recombination visualized by tdTomato fluorescence on tissue sections. If Western blotting confirms that *Crect;Ntn1*$^{CE/+}$ ears have less ectopic Netrin-1-myc protein, then this would support the idea that *FoxG1-Cre;Ntn1*$^{CE/+}$ animals are expressing an inhibitory level of ectopic Netrin-1-myc. *FoxG1-Cre;Ntn1*$^{CE/CE}$ homozygous embryos survive at least until E14.5, and in the future, paintfilling can be used to determine the effects of expressing two copies of the *Ntn1*^{CE} allele upon canal formation. Based on our findings, we expect that *FoxG1-Cre;Ntn1*$^{CE/CE}$ ears will exhibit canal truncation, but with a lower penetrance than *FoxG1-Cre;Ntn1*$^{CE/+}$ ears.

The effects of different genetic backgrounds may also explain the low penetrance of canal truncations in *FoxG1-Cre;Ntn1*$^{CE/+}$ ears. While lateral canal truncation is 100% penetrant in *Lrig3* mutants, this is only the case in a highly inbred C57BL/6 genetic background (Abraira et al., 2008). In fact, in a slightly outbred background, lateral canal truncation in *Lrig3* mutants is only 33% penetrant (del Rio et al., 2013). This suggests that canal formation can still proceed normally in the presence of ectopic *Netrin-1* and agrees with evidence that multiple factors are involved in canal formation. The *Ntn1*^{CE} allele was created using C57BL/6 blastocysts and ES cells derived from the 129S4/SvJae strain. In the future, backcrossing the *Ntn1*^{CE} line onto a C57BL/6 background will allow us to test whether genetic background differences underlie the low penetrance of canal truncations in *FoxG1-Cre;Ntn1*$^{CE/+}$ ears that we report here.

If *Ntn1*$^{CE/+}$ mice on a highly inbred C57BL/6 background still exhibit a lower penetrance of canal truncation defects compared to *Lrig3* mutants, it would suggest that loss of *Lrig3* causes canal truncation via other factors in addition to expanded *Netrin-1.*

While loss of one copy of *Netrin-1* was sufficient to rescue canal truncation in *Lrig3* mutants, it is likely that other factors downstream of *Lrig3* contribute to canal formation (Abraira et al., 2008). In particular, Lrig3 can bind to the FGF receptor tyrosine kinase *in vitro* and inhibits FGF signaling *in vivo* during *Xenopus* neural crest development (Zhao et al., 2008). As FGFs are well-established regulators of canal formation (Chang el al., 2004; Pirvola et al., 2004; Pauley et al., 2003), the *Lrig3* phenotype may also result from defects in FGF signaling. By expressing *Netrin-1* specifically in the lateral canal using *Otx1-Cre* (Puelles et al., 2003) we may be able to replicate the restricted ectopic *Netrin-1* expression observed in *Lrig3* mutants without any confounding effects *Lrig3* loss. The loss of endogenous *Netrin-1*, however, is known to lead to expanded *Lrig3 (*Abraira et al., 2008); if exogenous *Netrin-1* expression leads to downregulation of *Lrig3*, it will prove difficult to determine whether the effects of *Lrig3* loss are solely due to expanded *Netrin-1*.

Initially, we used forebrain lysate to compare levels of endogenous and exogenous Netrin-1 protein from *FoxG1-Cre;Ntn1*$^{CE/+}$ and *Ntn1*$^{CE/+}$ animals due to the abundance of forebrain tissue. While production of myc-tagged exogenous Netrin-1 was evident in *FoxG1-Cre;Ntn1*$^{CE/+}$ lysate, we were unable to detect the substantial increase in total Netrin-1 protein that would be expected when ectopic *Netrin-1* expression is driven using a strong CAG promoter. This may suggest that Netrin-1 is autoregulated post-translationally, and underscores the importance of carefully titrated Netrin-1 levels *in vivo*.

Netrin proteins are highly conserved. For instance, chick and mouse Netrin-1 are 89% identical (Serafini et al., 1996) and chick Netrin-1 and Netrin-2 can elicit axon

outgrowth from rat spinal cord explants (Serafini et al., 1994; Kennedy et al., 1994). As chick Netrins were the first vertebrate family members cloned (Serafini et al., 1999), most *in vitro* studies of Netrin function use chick Netrin-1 (i.e. Mehlen et al., 1998). The one previous study of exogenous *Netrin-1* expression in mice also used chick *Netrin-1* under the control of a gut specific promoter (Mazelin et al., 2004). Therefore, to enable comparison with previous studies, we also used chick *Netrin-1* in the creation of our conditional expression allele.

As we describe in Chapter 2, overexpression of chick *Netrin-1* in the developing chick inner ear interferes with fusion during canal formation. This is the opposite of the excessive fusion and canal truncation observed when endogenous *Netrin-1* is expressed ectopically in *Lrig3* mutants (Arbraira et al., 2008), and in fact, resembles the failure of fusion reported with loss of *Netrin-1* in mice (Salminen et al., 2000). We suggest in Chapter 2 that Netrin-1 has divergent cellular effects during canal formation. An alternate explanation, however, is that these differences are due to variation in the amino acid sequence of chick and mouse Netrin-1 protein, despite 89% identity (Salminen et al., 1996). The use of chick *Netrin-1* in our conditional allele allowed us to disprove this possibility: if the failure of canal fusion in chicks was caused by differences in the chick Netrin-1 protein, then expression of chick Netrin-1 protein in the mouse should also prevent fusion. The fact that ectopic expression of exogenous chick *Netrin-1* in the mouse caused canal truncation, the same defect observed when endogenous mouse *Netrin-1* is expressed ectopically in *Lrig3* mutants (Abraira et al., 2008), suggests that cellular responses to Netrin-1 are in fact different between chicks and mice.

In the future, the *Ntn1*^{CE} mouse can be used to test whether tissues outside of the inner ear are sensitive to the effects of Netrin-1 on the basement membrane. The requirement of Unc6 for basement membrane breakdown during vulval development in worms (Ziel et al, 2009) supports a conserved role for Netrin in basement membrane regulation. Furthermore, Netrin proteins have been observed at the basement membrane in several tissues undergoing branching morphogenesis, a process that involves basement membrane remodeling. In fact, loss of *Netrin-1* disrupts branching morphogenesis in both the pancreas (Yebra et al., 2003) and the mammary gland (Srinivasan et al., 2003), thus these tissues would be a good first place to test if Netrin-1 influences basement membrane integrity in other tissues.

If Netrin-1 has a general ability to modulate the basement membrane, this would raise the intriguing possibility that other aspects of Netrin-1 function are also influenced by its effects on the basement membrane. For example, Netrin-1 has already been shown to act as a survival factor for tumor cells (reviewed in Mehlen et al., 2011) and increased *Netrin-1* expression has been observed in many cancers (Fitament et al., 2008; Delloye-Bourgeois et al., 2009; Link et al., 2007). If Netrin-1 can regulate basement membrane integrity, in addition to providing a survival advantage, increased Netrin-1 could allow tumor cells to cross the vascular basement membrane during metastasis.

Chapter 4: Creation of a floxed *Netrin-1* allele in mouse

Allison M. Nishitani, Andrea Yung, and Lisa V. Goodrich

A.M. Nishitani created the floxed allele of *Netrin-1* and analyzed canal formation in *Netrin-1* mutants. A. Yung performed the Robo3 immunostaining to characterize the defects in spinal cord commissure formation. Blastocyst injections were performed by the Boston Children's Hospital transgenic core. The *Crect* line was provided by Trevor Williams, University of Colorado Denver.

Introduction

The semicircular canals of the inner ear form from epithelial pouches which fuse in the center to create the mature canal structure. A key step in this process is the localized breakdown of the basement membrane in the region of the epithelium that undergoes fusion. *Netrin-1* is specifically expressed in the fusion plate epithelium, and in *Netrin-1* mutants, the basement membrane fails to break down and fusion does not occur, leaving the animals with unfused pouches instead of canals (Salminen et al., 2000). Conversely, in mice with expanded *Netrin-1* expression as a result of *Lrig3* loss, basement membrane breakdown and fusion occur early and in an unrestricted manner, leading to canal truncation (Abraira et al., 2008). Further, in Chapter 3 we show that direct expression of *Netrin-1* is sufficient to cause canal truncation, providing more evidence that the main cellular effect of Netrin-1 during canal formation in mice is to regulate basement membrane integrity and fusion. Despite this, the mechanism by which Netrin-1 regulates basement membrane integrity remains unknown.

There is growing evidence that Netrin-1 can regulate the basement membrane. Netrins are capable of binding extracellular matrix components including heparin sulfate proteoglycans (Geisbrecht et al., 2003; Kappler et a., 2000), laminin (Schneiders et al., 2007), and type IV collagen (Yebra et al., 2003) and localize to the basement membrane during the morphogenesis of multiple tissues including the lung (Liu et al., 2004), salivary gland (Schneiders et al., 2007), and pancreas (Yebra et al., 2003). Unc6 has also been shown to be required for basement membrane breakdown in the worm during vulvar development, suggesting that basement membrane regulation by Netrins may be an evolutionarily conserved function (Ziel et al., 2009).

Based on the accumulating evidence linking Netrin-1 to the basement membrane in multiple systems, we wondered if Netrin-1 acts to regulate basement membrane integrity in other tissues outside of the developing inner ear. In the developing lung, pancreas, mammary gland, and salivary gland, for example, Netrins are present in the basement membrane and are important for branching morphogenesis (Liu et al., 2004; Yebra et al., 2003; Srinivasan et al., 2003; Schneiders et al., 2007). Although defects in basement membrane integrity outside the developing inner ear have not been reported, they may have been overlooked for several reasons. For example, it is possible that defects in basement membrane regulation in other tissues will only be observed in a true null. Existing *Netrin-1* hypomorphs contain a small amount of wild type transcript (Serafini et al., 1996), which may be sufficient to properly regulate the basement membrane in tissues outside of the inner ear. Further, some of the candidate tissues where Netrin-1 may play a role in basement membrane regulation develop after birth (i.e. mammary gland) and cannot be investigated because existing *Netrin-1* mutants die neonatally. To overcome the problem of early lethality and to generate a true *Netrin-1* null, we generated a conditional knockout allele of *Netrin-1*. This will expand our ability to study the role of Netrin-1 in basement membrane regulation and other processes.

Netrin is a multifunctional molecule best known for its role in axon guidance during neurodevelopment. Despite the intensive study of Netrin-1 over the last twenty years, some ambiguity about Netrin-1 function *in vivo* remains. A classic function of Netrin-1 is the guidance of commissural axons to the midline. This role has been confirmed *in vitro*, where Netrin-1 promotes outgrowth of commissural axons from spinal cord explants (Kennedy et al., 1994; Serafini et al., 1994), and *in vivo*. When Netrin-1

levels are severely reduced, few commissural axons reach the ventral spinal cord and the ventral commissure is greatly reduced (Serafini et al., 1996). In addition to its attractive roles, *in vitro* data supports a role for Netrin in repelling populations of cells away from the midline. For example, Netrin expressing floorplate explants repel trochlear motor neuron axons *in vitro* and COS cells expressing recombinant Netrin-1 have the same effect, although to a lesser degree (Colamarino and Tessier-Lavigne, 1995). However, in *Netrin-1* hypomorphs, however, trochlear motor axon guidance is relatively normal (Serafini et al., 1996), suggesting that other floorplate cues guide trochlear projections away from the midline. The presence of residual wild type *Netrin-1* transcript in these hypomorphs complicates definitive conclusions about the role of Netrin-1 in repelling trochlear motor axons *in vivo*.

Another unresolved issue is that the phenotypes uncovered in existing *Netrin-1* mutants poorly reflect the sum of the receptor phenotypes. In particular, only a subset of phenotypes described in Unc5 receptor mutants are present in *Netrin-1* hypomorphs. For example, *Unc5a* null animals have decreased apoptosis in the cervical spinal cord at E12, during a period of normal motor neuron cell death, while *Netrin-1* hypomorphs exhibited similar levels of cell death as wild type animals (Williams et al., 2006). Similarly, *Unc5b* mutants have severe vascular defects that cause lethality at E12.5 (Lu et al., 2004), whereas *Netrin-1* hypomorphs are viable until birth, suggesting that Unc5b has functions independent of Netrin-1. Finally, *Unc5c* mutants have trochlear and phrenic nerve guidance defects, which are absent in *Netrin-1* hypomorphs (Burgess et al., 2006). The creation of a null allele of *Netrin-1* will allow us to determine if the

differences between phenotypes in the *Netrin-1* hypomorphs and receptor mutants are due to alternative ligands or residual Netrin-1.

Finally, Netrin-1 expression is known to be maintained in the adult nervous system (reviewed in Manitt and Kennedy, 2002) and some *in vitro* evidence suggests that Netrin may play a role in cortical synaptogenesis (Goldman et al., 2013). However, as existing *Netrin-1* mutants die shortly after birth, the role of Netrin-1 in the adult nervous system remains unclear.

This chapter summarizes the creation and validation of a new floxed allele of *Netrin-1*. Initial analysis focused on null animals created by crossing the floxed allele to animals with Cre active in the germline. *Netrin-1* null tissue was used to confirm a loss of Netrin-1 protein and to assess phenotypes described in the original *Netrin-1* hypomorphic mutants in the developing nervous system and morphogenesis of the inner ear. In the future, this mouse line can be used to assess whether Netrin-1 regulates the basement membrane outside of the inner ear, as well as to define definitive *in vivo* functions for Netrin-1.

Materials and methods

Mice

The *Netrin-1* hypomophic genetrap line has been previously reported (Serafini et al., 1996). It contains an insertion of the secretory gene trap vector pGT1.8TM within the second intron of the *Netrin-1* gene. The gene trap was designed to capture the N-terminal signal sequence of an endogenous gene and create an insertion of beta-galactosidase to generate a truncated version of the target protein fused to the reporter

(Skarnes et al., 1995). *Ntn1* gene trap mice were maintained as heterozygotes and genotyping was performed using an X-gal reaction to detect the presence of lacZ in tissue samples. *Ntn1*$^{trap/+}$ mice were crossed to generate homozygous *Ntn1*$^{trap/trap}$ embryos, which were genotyped using a real time PCR system to detect lacZ copy number (Transnetyx). This line has been maintained for over ten generations on the C57BL/6 background.

As described in this chapter, the floxed *Netrin-1* line was generated by homologous recombination using a targeting construct with loxP sites flanking the ATG containing exon 3 of *Netrin-1*. Genotyping was performed using primers which flank the first loxP site:

AMN324(GGCCAGGCAAACTTTTCTTT)

AMN328(AGGGGCTTCCAAACCTGAA)

A 319 bp product is generated in the absence of the loxP site, while a 353 bp product is generated in the presence of the loxP site. These primers allow for the detection of homozygous *Ntn1*$^{fl/fl}$ animals. To remove the neo cassette, *Ntn1*$^{floxed\text{-}neo/+}$ mice were crossed to a global FLPe driver (JAX, B6;SJL-Tg(ACTFLPe)9205Dym/J) (Rodriguez et al., 2000). Removal of neo was detected by a real time PCR system (Transnetyx).

As reported in this chapter, a null allele of *Netrin-1* was created by crossing Ntn1$^{fl/+}$ animals to a global Cre active in the germline (JAX, B6.FVB-Tg(EIIa-cre)C5379Lmgd/J) (Lasko et al., 1996). Genotyping was performed using a pair of primers that span the deleted exon which generate a 432 bp product if exon 3 has been removed:

AMN363(CAGGTGGCAAGAGAAAAGGA)

AMN437(TCCGTTTGGATCTGGGATTA)

In combination with a pair of primers inside the deleted exon that generates 468 bp product if exon 3 is present, we can detect homozygous *Ntn1*$^{-/-}$ animals:

AMN357(CTCAATAACCCGCACAACCT)

AMN358(CTCCGAGTCGTCTTCGTTCT)

Targeting construct creation

For the following descriptions, the ATG is designated as position 1 in the *Netrin-1* genomic locus. PCR using a BAC template generated a 2855 bp fragment spanning base pairs -1693 to +1161 in the *Netrin-1* locus, which was sub-cloned into the pCR II-TOPO vector. Restriction sites were added in the primers to allow future cloning (NdeI 5' and SalI 3'). A linker sequence containing a loxP site and an AccI site was inserted into an AflII site upstream of exon 3 at position -349 bp. PCR using a BAC template generated a 3451 bp fragment spanning base pairs +1142 to +4592 in the *Netrin-1* locus, which was sub-cloned into the pCR II-TOPO vector. Restriction sites were added in the primers to allow future cloning (XhoI 5' and ClaI 3'). These genomic fragments were then cloned into the 4600c vector to create the floxed *Ntn1* targeting construct using NdeI/SalI for the 5' fragment and XhoI/ClaI for the 3' fragment. The 4600c vector contains a *loxP* sequence and an *frt*-flanked *neomycin* cassette (Yu et al., 2013).

The 5' arm was amplified from a BAC template (bMQ371-c23) using the following primers:

AMN1 (TACAACCATATGTTGGGAAAGTTATCCCTAGCC)

AMN5 (TAAGTCGACTAAGGTCTCCGAAACCCACT).

Likewise, the 3' arm was amplified from a BAC template (bMQ371-c23) using the following primers:

AMN6 (TACCTCGAGAGTGGGTTTCGGAGACCTTA)

AMN7 (TACAACATCGATGCGTCTTTTGTGTGGGTTTT).

The linker sequence used to insert the 5' loxP site was created by annealing the following oligos to create a linker with AflII sticky ends:

AMN174 (TTAAGTAGACATAACTTCGTATAATGTATGCTATACGAAGTTAT)

AMN175 (TTAAATAACTTCGTATAGCATACATTATACGAAGTTATGTAGAC)

ES cell screening

The 5' AccI probe sequence (corresponding to position -3377 to -3060 bp in the *Netrin-1* locus) was synthesized and subcloned into the pUC57 vector using flanking XhoI sites. XhoI was used to excise the probe fragment, which was gel purified using the GeneClean III kit (MP Biomedical). The probe was radiolabeled using a random primed DNA labeling kit (Stratagene).

The 3' HindIII probe sequence (corresponding to position +6747 to +7687 bp in Netrin-1 locus) was amplified from BAC DNA using the following PCR primers: AMN25(GTTGCAATGCAGACGTTCC) and AMN26(ACCCAGCAGTCCAGTCTCAC) and was cloned into the pCRII-TOPO vector. EcoRI sites flanking the TOPO cloning site

were used to excise the probe fragment, which was gel purified using the GeneClean III kit (MP Biomedical). The probe was radiolabeled using a random primed DNA labeling kit (Stratagene).

To verify the Cre-mediated excision of exon 3, ES cells were transfected with Cre. Genomic DNA was extracted and PCR primers spanning exon 3 were used to detect the removal of the exon in the presence of Cre:

AMN309 (CGTTGAGACAGGACGCTCTT)

AMN310 (TATCCGCTCACAATTCCACA)

These primers generate a product of 2012 bp in the absence of recombination and a product of 497 bp after removal of exon 3.

Paintfilling

E14.5 heads were fixed overnight in Bodian fix at 4°C, washed for 10 minutes in 100% ethanol, and dehydrated overnight in 100% ethanol at room temperature. Samples were then rinsed briefly in methyl salicylate and cleared overnight in methyl salicylate at room temperature. Heads were hemisected along the midline and their cochleae were injected from the medial side with whiteout diluted to 0.025% with methyl salicylate using a pulled glass pipette and a Hamilton syringe.

Western blot

E11.5 heads were lysed using an electronic homogenizer and 200 µl of the following lysis buffer: 50 mM tris pH7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycolate, 0.1% SDS, 1x Pefabloc SC PLUS protease inhibitor (Roche). The following primary antibody was used: Netrin-1 (1:500, R&D MAB1109).

Immunohistochemistry

Embryonic tissue was fixed overnight at 4°C in 4% PFA/PBS and washed 3 times for 10 minutes in PBS. Tissue was then put through a sucrose series (10% sucrose/PBS, 20% sucrose/PBS, 30% sucrose/PBS) and transitioned into Neg-50 embedding media (Richard-Allen Scientific) with incubation in each solution overnight at 4°C. Tissue was embedded in Neg-50 by freezing with a slurry of dry ice and isopentane.

20 µm cryosections were blocked for one hour at room temperature using 3% BSA in PBS and permeabilized for 15 minutes at room temperature using 1% BSA and 0.1% TritonX-100 in PBS. Sections were incubated overnight at 4°C with a primary antibody that detects Robo3 (1:100, R&D) diluted in permeabilization solution. The following day, sections were incubated at room temperature for 1.5 hours in secondary antibody (1:750, Alexa Fluor 568, Life Technologies) diluted in permeabilization solution. Sections were counterstained with DAPI (1:10,000) and mounted using Fluoromount-G (Southern Biotech).

Results

Creation of floxed Netrin-1 mice

We chose to delete the third exon of Netrin-1, which encodes the ATG, signal sequence, and the majority of the N-terminus of the Netrin-1 protein. The absence of the signal sequence should ensure that any protein generated from an alternate start site is not secreted (Figure 4.1). The floxed *Netrin-1* targeting construct (Figure 4.2 B) was created using the 4600c vector, which contains a *loxP* sequence, *frt*-flanked *neomycin* cassette for selection, and 5' and 3' multiple cloning sites for insertion of 5' and 3' homologous arms (see Materials and Methods for details).

The targeting construct was linearized using AscI and electroporated into J1 ES cells (derived from 129S4/SvJae strain) and selected under G418 for 1 week. 192 ES cell clones were screened for correct recombination by Southern blot using external 5′ and 3′ probes. To screen for 5' recombination, DNA was digested with AccI and probed with radiolabeled DNA corresponding to position -3377 to -3060 bp within the *Netrin-1* locus (Figure 4.2 A). Homologous recombination results in a band shift from 5 kb to 3 kb due to the introduction of an AccI site in the 5' loxP site (Figure 4.2 B). To screen for 3' recombination, DNA was digested with HindIII and probed with radiolabeled DNA corresponding to position +6747 to +7687 bp within the *Netrin-1* locus (Figure 4.2 B). Homologous recombination results in a band shift from 10.8 kb to 8.3 kb due to the introduction of a HindIII site in the neomycin cassette (Figure 4.2 B). Three clones (#30, 110, and 165) were positive for 5' and 3' recombination (Figure 4.2 C). Karyotyping revealed that less than 30% of cells from clone 30 had the normal number of chromosomes. Although 90% of cells from clone 165 had a normal number of

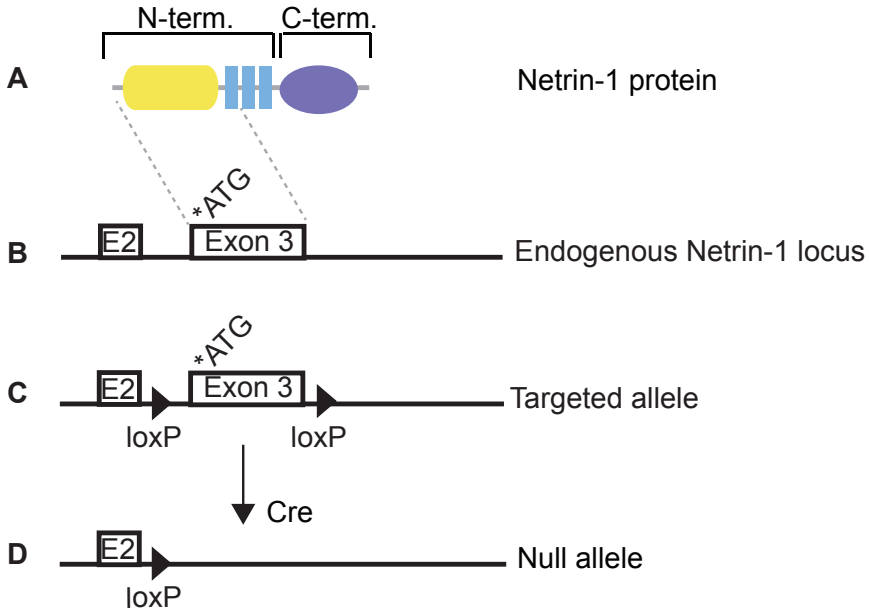


Figure 4.1: Design of the *Ntn1 floxed* allele. A) The N-terminus of Netrin-1 is composed of domains VI (yellow) and V (blue), which are related to laminin. The C-terminus is composed of an NTR domain (purple). B) The ATG, signal sequence, and the majority of the N-terminus of the Netrin-1 protein are encoded in exon 3. C) The targeted allele was created by floxing exon 3 of Netrin-1 with loxP sites. D) Upon Cre mediated recombination, removal of exon 3 is predicted to create a null due to the lack of signal sequence and ATG.

chromosomes, the wild type band was much stronger than the recombined band on the Southern blot, suggesting a mixed clone. Clone 110, on the other hand, had a strong recombined band (Figure 4.2 C) and 80% of cells showed a normal number of chromosomes. Therefore, we chose to use these cells for blastocyst injection. To verify Cre mediated removal of exon 3, we transfected ES cells from clone 110 with Cre-GFP, GFP, or no DNA. The deletion of exon 3 was detected by PCR primers that span the third exon and generate a 497 bp band if the exon has been deleted. In ES cells from clone 110, this PCR product was present only in the presence of Cre, confirming that the floxed exon can be successfully removed (Figure 4.2 D).

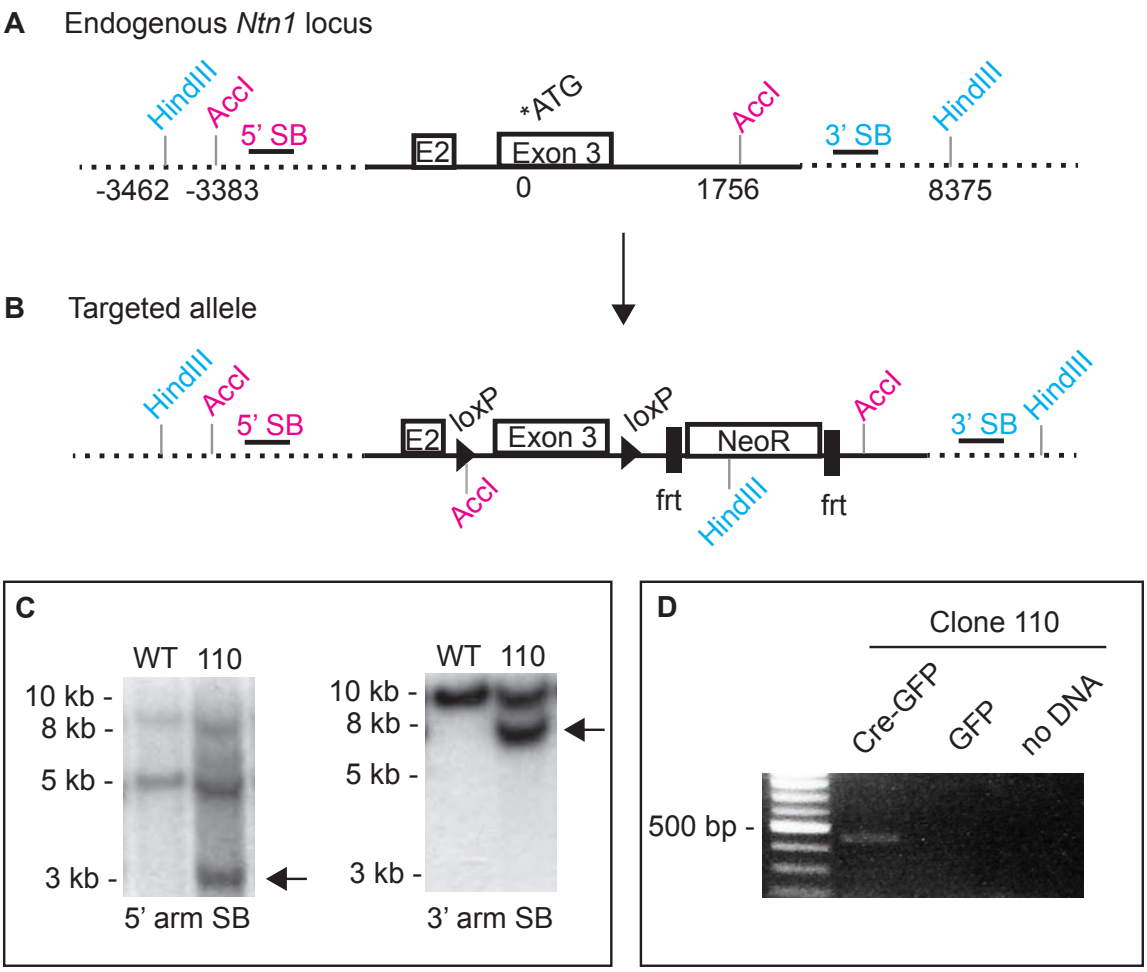
4 chimeric males were born and PCR genotyping was used to detect the presence of the floxed allele in their progeny. PCR primers spanning the first loxP site detect the presence of the floxed allele. Germline transmission occurred in 3 of the chimeric males, yielding *Ntn1*$^{floxed\text{-}neo/+}$ mice. *Ntn1*$^{floxed\text{-}neo/+}$ mice were mated to a global *FLPe* driver (JAX, B6;SJL-Tg(ACTFLPe)9205Dym/J) (Rodriguez et al., 2000) to remove the neo cassette, creating *Ntn1*$^{fl/+}$ mice. FLPe was then bred out and *Ntn1*$^{fl/fl}$ homozygotes were created. *Ntn1*$^{fl/fl}$ mice are viable and fertile.

Ntn1$^{fl/+}$ mice were crossed to a global Cre active in the germline (JAX, B6.FVB-Tg(EIIa-cre)C5379Lmgd/J) (Lasko et al., 1996) to create a null allele. Unlike *Ntn1*$^{trap/trap}$ hypomorphs, Netrin-1 protein was undetectable in E11.5 head lysate from *Ntn1*$^{-/-}$ animals, suggesting that they represent a true *Netrin-1* null (Figure 4.3). Like hypomorphic genetrap animals, *Ntn1*$^{-/-}$ animals die shortly after birth.

To determine if the complete loss of Netrin-1 protein could generate more severe phenotypes than those observed in *Netrin-1* hypomorphs, we initially assessed the best

Figure 4.2: Floxed Ntn1 ES cell screening. A) The WT Netrin-1 locus and B) the targeted floxed Netrin-1 allele with exon 3 flanked by loxP sites and an added frt flanked neomycin cassette for selection. The translational start site of the Netrin-1 coding sequence is designated as position 0. ES cell clones were screened by Southern blot using external 5' and 3' probes. To screen for 5' recombination, DNA was digested with AccI and probed with radiolabeled DNA (5' SB) within the Netrin-1 locus. C) Homologous recombination results in a band shift detected by Southern blot from 5 kb to 3 kb (WT vs. ES cell clone 110) due to the introduction of an AccI site in the 5' loxP site. To screen for 3' recombination, DNA was digested with HindIII and probed with radiolabeled DNA (3' SB) within the Netrin-1 locus. Homologous recombination results in a band shift detected by Southern blot from 10.8 kb to 8.3 kb (WT vs. ES cell clone 110) due to the introduction of a HindIII site in the neomycin cassette. D) After Southern blot screening, ES cell clone 110 was tested for removal of the floxed exon 3 upon introduction of Cre. ES cells were transfected with Cre-GFP, GFP, or no DNA. Genomic DNA was collected and PCR was performed using primers the span the third exon. If exon 3 has been excised, the PCR will generate a 497 bp product while if exon 3 remains, the PCR will generate a 2012 bp product. The 497 bp product was observed in the presence of Cre, indicating removal of the floxed exon 3.

Figure 4.2 (Continued)



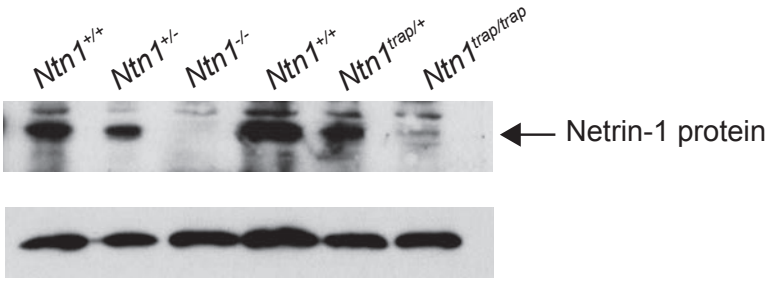


Figure 4.3: Loss of Netrin-1 protein in *Ntn1*$^{-/-}$ animals. *Ntn1*$^{fl/+}$ animals were crossed to EIIa-Cre animals to remove *Ntn1* from the germline and create *Ntn1*$^{+/-}$ animals. Like previously reported *Ntn1*$^{trap/trap}$ hypomorphs, *Ntn1*$^{-/-}$ homozygoous animals die shortly after birth. E11.5 head lysate was prepared for western blot analysis from the newly generated *Ntn1*$^{-/-}$ mice and the previously reported *Ntn1* genetrap hypomorphic mice. Blotting with an anti-Netrin antibody reveals a complete loss of Netrin-1 protein in *Ntn1*$^{-/-}$ lysate, while a small amount of Netrin-1 protein is detectable in the *Ntn1*$^{trap/trap}$ lysate.

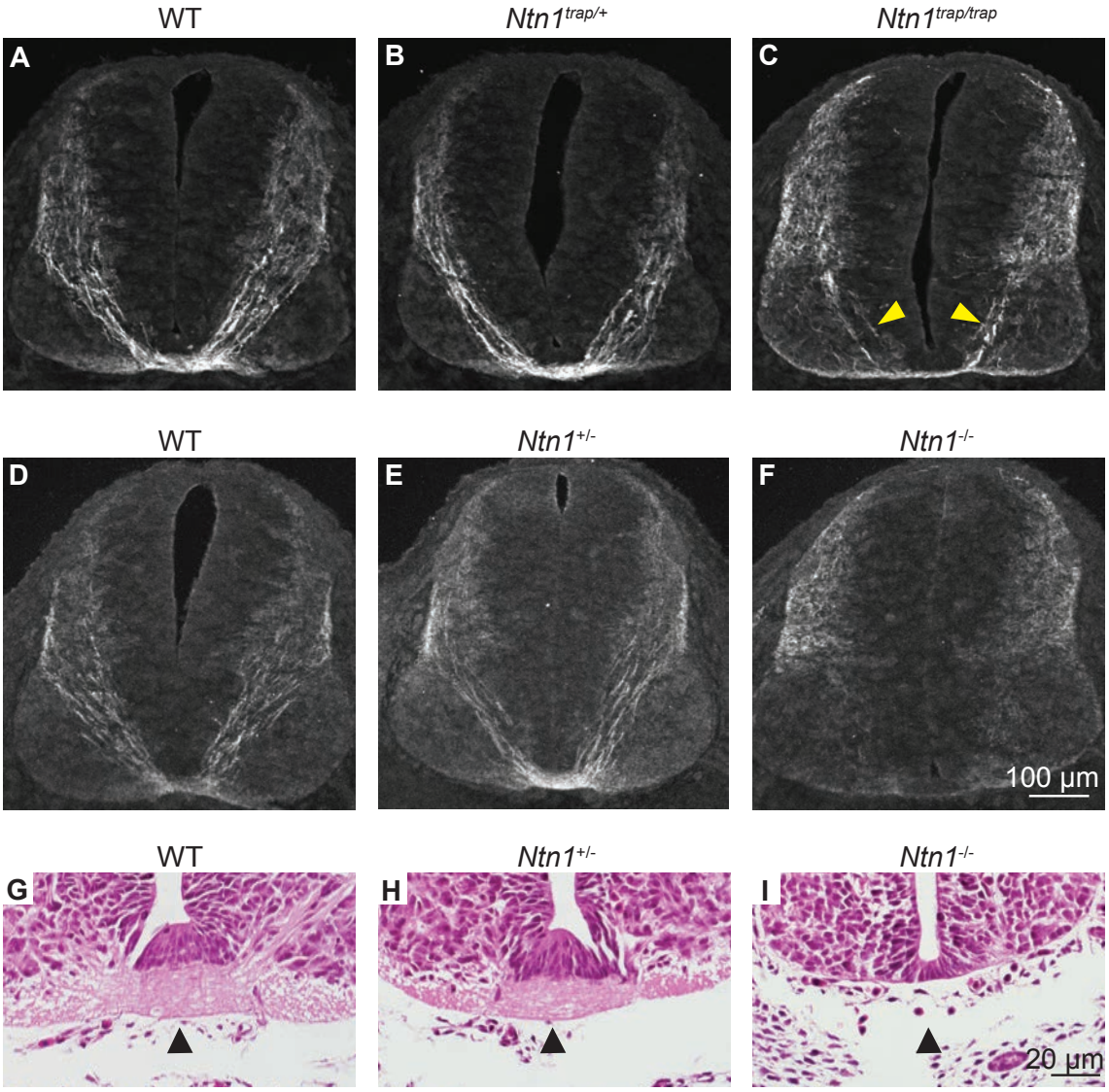
described defect in *Ntn1* hypomorphic mutants: commissural axon guidance in the developing spinal cord. Robo3 antibody staining to mark commissural axons in E11.5 spinal cord tissue sections revealed that very few, if any, commissural axons reach the midline in *Ntn1*$^{-/-}$ embryos (Figure 4.4 F). H&E staining also revealed the complete lack of a ventral commissure in *Ntn1*$^{-/-}$ spinal cords (Figure 4.4 I). Previously reported hypomorphic mutants have greatly reduced commissural axon crossing (Figure 4.4 C) which was hypothesized to be mediated by other axon guidance factors. Our data suggests instead that Netrin-1 may be required for all commissural axon guidance to the midline.

The effects of Netrin-1 loss on canal morphogenesis

In the mouse, *Netrin-1* is expressed beginning at E10.5 in the region of the otic epithelium that will form the fusion plate (Salminen et al., 2000; Matilainen et al., 2007). *Netrin-1* expression continues at the fusion plate throughout canal formation and it remains expressed in the canal rim postnatally (Salminen et al., 2000). In *Netrin-1* hypomorphs, semicircular canal formation is severely disrupted: the posterior and lateral canals fail to form, and the anterior canal only sometimes partially fuses (Salminen et al., 2000). *Netrin-1* expression is not restricted to the semicircular canals. At E13.5, *Netrin-1* is expressed in the non-sensory areas of the developing saccule and utricle, as well as in the developing cochlea. By E17.5, *Netrin-1* expression in the cochlea is restricted to Reissner's membrane, while expression is maintained in the non-sensory areas of the utricle and saccule (Salminen et al., 2000).

Figure 4.4: Very few commissural axons cross the midline in *Ntn1*$^{-/-}$ mutants. A-F) Robo3 staining on E11.5 spinal cord tissue sections identifies commissural axons. In wild type littermate controls (A) and heterozygotes for the hypomorphic *Ntn1*$^{trap/+}$ (B), commissural axons cross the midline. In hypomorphic *Ntn1*$^{trap/trap}$ mutants, far fewer axons reach the midline (arrowheads, C). In wild type littermate controls (D) and *Ntn1*$^{+/-}$ heterozygotes (E), commissural axons cross the midline. In *Ntn1*$^{-/-}$ mutants, very few, if any, commissural axons cross the midline and instead invade the ventricular zone and motor columns (F). G-I) H&E staining of E11.5 spinal cord tissue sections. While the ventral commissure is present in wild type littermate controls (arrowhead, G) and *Ntn1*$^{+/-}$ heterozygotes (arrowhead, H), the ventral commissure is absent in *Ntn1*$^{-/-}$ mutants (arrowhead, I).

Figure 4.4 (Continued)

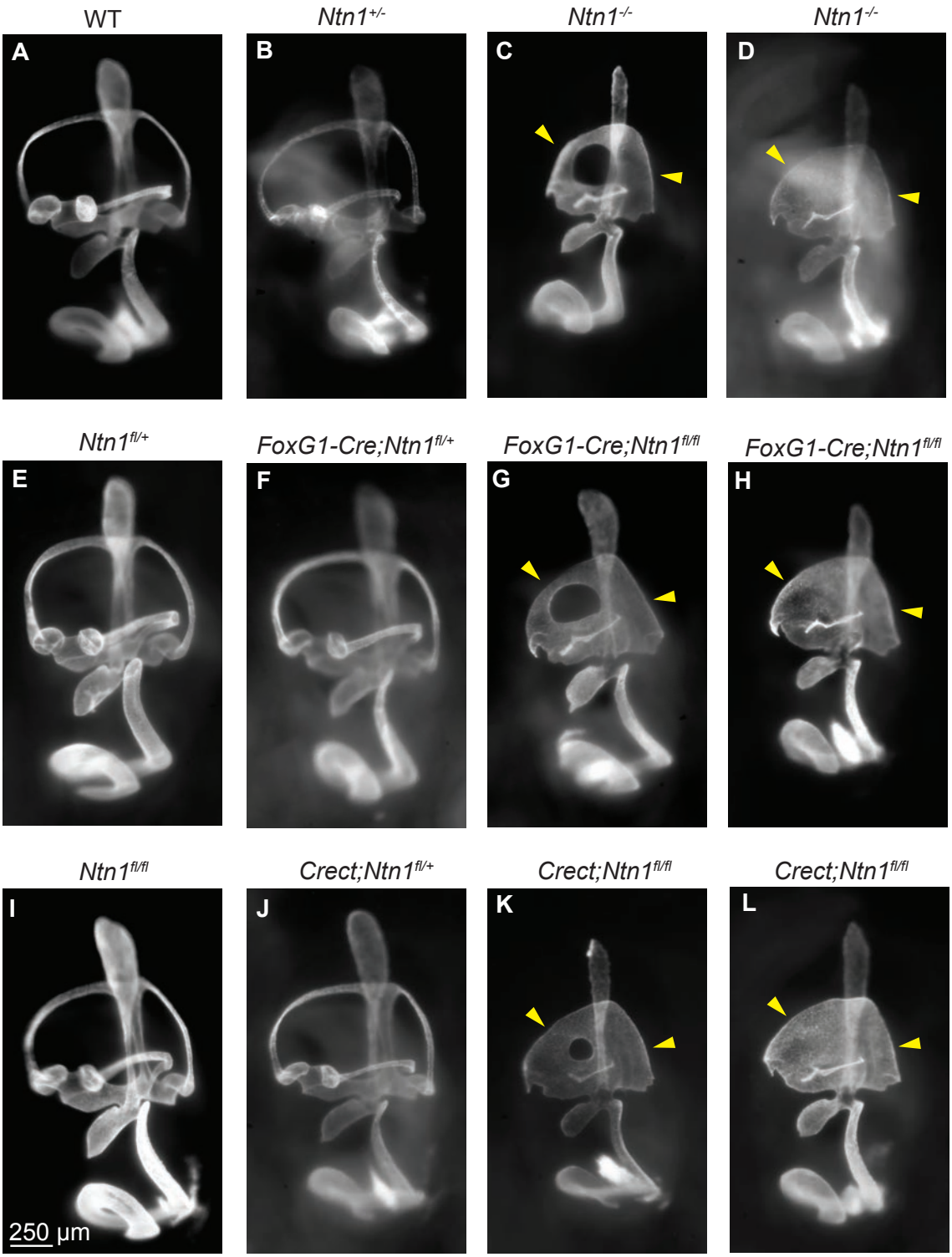


To assess whether complete loss of Netrin-1 protein causes additional and/or more severe defects in gross inner ear morphogenesis, inner ear morphology was assessed by paintfilling E14.5 *Ntn1*$^{-/-}$ ears (Figure 4.5). *Ntn1*$^{+/-}$ control ears were normal (Figure 4.5 B, n=6) while all *Ntn1*$^{-/-}$ ears exhibited defects in canal formation (Figure 4.5 C and D, n=8). Similar to previously reported *Ntn1* hypomorphs (Salminen et al., 2000), *Ntn1*$^{-/-}$ ears either had a partially fused anterior canal (Figure 4.5 C, n=4) or a complete lack of fusion (Figure 4.5 D, n=4). Thus, at least at the gross morphological level, the complete loss of Netrin-1 protein did not reveal more severe or additional phenotypes in the developing inner ear compared to existing *Ntn1* hypomorphs.

The same analysis was performed using the floxed allele of *Netrin-1* with two different Cre drivers: FoxG1-Cre and Crect. As described in Chapter 3, FoxG1-Cre is active in the entire otic epithelium while Crect drives salt-and-pepper recombination in the otic epithelium. *Ntn1*$^{fl/fl}$ mice exhibit normal canal morphology (Figure 4.5 I, n=6). Similarly, removing one copy of *Netrin-1* using FoxG1-Cre (Figure 4.5 F, n=7) or Crect (Figure 4.5 J, n=4) did not affect canal formation. As expected, *FoxG1-Cre;Ntn1*$^{fl/fl}$ mice phenocopied *Ntn1*$^{-/-}$ mice: all ears examined exhibited either a partially fused anterior canal (Figure 4.5 G, n=3) or a lack of fusion (Figure 4.5 H, n=3). Sparser recombination in *Crect;Ntn1*$^{fl/fl}$ mice resulted in an identical phenotype, with all ears exhibiting either a partially fused anterior canal (Figure 4.5 K, n=1) or a lack of fusion (Figure 4.5 L, n=7). This suggests that low levels of Netrin-1 protein are not sufficient to regulate fusion, and fits data suggesting that canal formation is impaired similarly between *Netrin-1* hypomorphs and *Netrin-1* nulls.

Figure 4.5: Loss of Netrin-1 disrupts canal formation. Paintfilling at E14.5 reveals that the loss of Netrin-1 disrupts canal formation. *Ntn1*$^{+/-}$ heterozygote ears display normal canal morphology (B, n=6). However, *Ntn1*$^{-/-}$ ears display severe defects in canal formation with either only the anterior canal partially fusing (arrowheads C, n=4) or a complete lack of fusion (arrowheads D, n=4). The floxed allele replicates the null when crossed to FoxG1-Cre. *Ntn1*$^{fl/fl}$ (E, n=6) and *FoxG1-Cre;Ntn1*$^{fl/+}$ ears (F, n=7) display normal canal morphologies, while *FoxG1-Cre;Ntn1*$^{fl/fl}$ ears phenocopy *Ntn1*$^{-/-}$ ears with either only the anterior canal partially fusing (arrowheads G, n=3) or a complete lack of fusion (arrowheads H, n=3). The use of Crect with the floxed allele also replicates the null phenotype. *Crect;Ntn1*$^{fl/+}$ ears (J, n=4) display normal canal morphologies, while *Crect;Ntn1*$^{fl/fl}$ ears phenocopy *Ntn1*$^{-/-}$ ears with either only the anterior canal partially fusing (arrowheads K, n=1) or a complete lack of fusion (arrowheads L, n=7).

Figure 4.5 (Continued)

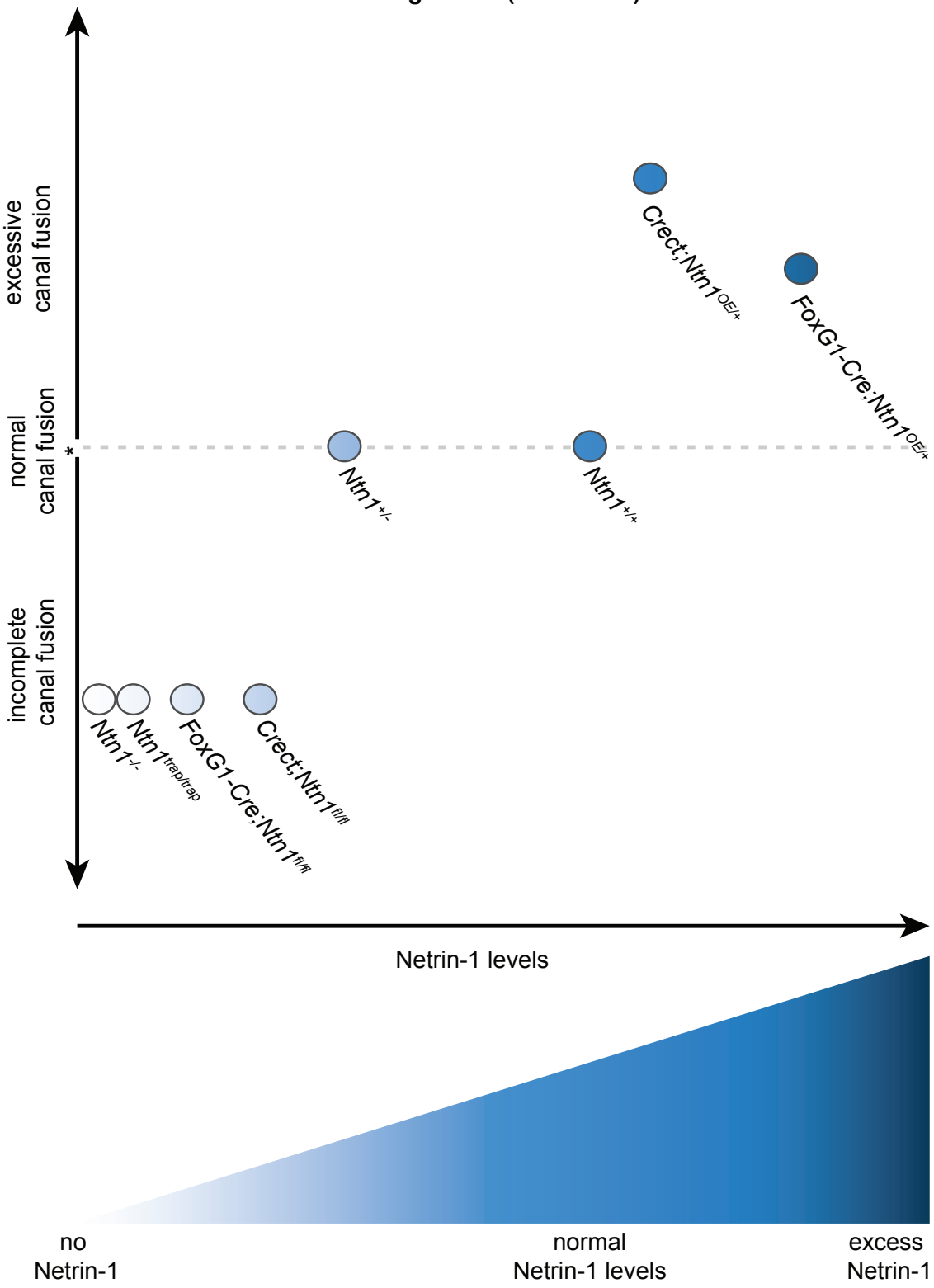


Combining the ectopic expression and loss of function data, we can begin to assemble a model describing the effects of different levels of *Netrin-1* expression on canal morphogenesis (Figure 4.6). In this chapter, we describe different genetic manipulations to remove *Netrin-1*. *Ntn1*$^{+/-}$ heterozygous ears form normally, but loss of *Netrin-1* from only a small subset of otic epithelial cells in *Crect;Ntn1*$^{fl/fl}$ ears resulted in a fully penetrant failure of fusion. Either a complete failure of fusion or partial fusion of only the anterior canal was observed in all of the following loss of function manipulations: *Ntn1*$^{-/-}$, *Ntn1*$^{trap/trap}$, *FoxG1-Cre;Ntn1*$^{fl/fl}$, *Crect;Ntn1*$^{fl/fl}$ (arranged presumably in order from least to most Netrin-1 protein present). There are two possible explanations for these observations. Either there is a threshold of Netrin-1 required throughout the tissue for fusion to proceed. Or, alternatively, complete loss of Netrin-1 from only some cells could sufficient to disrupt fusion altogether. To differentiate between these two possibilities it would be helpful to know the precise levels of overall Netrin-1 expression throughout the otic vesicle between these different genotypes. This experiment may help us to determine whether the relevant factor is the overall level of Netrin-1 in the tissue or if the special distribution of Netrin-1 expressing cells is more important.

The ectopic expression studies presented in Chapter 3 suggest that canal formation is also sensitive to increases in Netrin-1 levels. While inducing ectopic expression of *Netrin-1* always caused canal truncation, it seems that very high *Netrin-1* levels are inhibitory. Inducing expression throughout the otic epithelium using *FoxG1-Cre* caused canal truncation in 14% of *FoxG1-Cre;Ntn1*$^{CE/+}$ ears

Figure 4.6: Summary of loss of function and ectopic expression of *Netrin-1* on canal formation in the mouse. The mouse models used in this thesis comprise a series spanning from a complete lack of Netrin-1 to an excess of Netrin-1. This is schematized at the bottom of the plot as a gradient spanning from white (no Netrin-1) to dark blue (excess Netrin-1). The various genotypes examined in this work are color coded and plotted along the x-axis relative to the presumed amount of Netrin-1 present, from no Netrin-1 (*$Ntn1^{-/-}$*) to the most ectopic Netrin-1 (*FoxG1-Cre;$Ntn1^{OE/+}$*). The y-axis is a representation of canal fusion, with normal canal fusion marked by the dashed gray line. Above the dashed gray line is excessive canal fusion, while below the gray line is incomplete canal fusion.

Figure 4.6 (Continued)



(n=7/49 affected), while spare recombination in the otic epithelium using *Crect* caused canal truncation in 26% of *Crect;Ntn1*$^{CE/+}$ ears (n=9/34 affected). As *in* vitro assays demonstrate that Netrin-1 function is concentration dependent, with high levels of Netrin-1 inhibiting its activity, we predict that Netrin-1 function during canal morphogenesis is also concentration dependent. Examining canal morphology in *FoxG1-Cre;Ntn1*$^{CE/CE}$ ears, which should have even more induced *Netrin-1* expression, will help to solidify this hypothesis.

Discussion

Netrin-1 is involved in a number of important processes during development including axon guidance, cell migration, cell survival, and adhesion. Unfortunately, our understanding of Netrin-1 function *in vivo* is incomplete because existing *Netrin-1* mutants are non-conditional hypomorphs. We created a conditional *Netrin-1* mouse line to allow tissue-specific interrogation of Netrin-1 functions and to produce a true null allele to assess whether complete loss of Netrin-1 protein would reveal additional or more severe phenotypes.

The complete loss of Netrin-1 protein did not reveal additional or more severe defects in canal formation compared to hypomorphic *Ntn1*$^{trap/trap}$ mutants. All *Ntn1*$^{-/-}$ ears either lacked fusion or only had a partially fused anterior canal, as previously reported in *Netrin-1* hypomorphs (Salminen et al., 2000), suggesting that the small amounts of Netrin-1 protein remaining in the hypomorphs are not sufficient to affect fusion. The occasional partial fusion of the anterior canal in *Ntn1*$^{-/-}$ ears suggests that other mechanisms which enable fusion may be at play.

One possibility is that because the anterior canal fuses first and has the largest fusion plate, it is least susceptible to perturbations in the fusion process. Perhaps the anterior canal with its larger fusion plate uses multiple mechanisms to ensure proper fusion, and in the absence of Netrin-1 mediated basement membrane breakdown, a small amount of fusion can occur.

Importantly, the complete loss of Netrin-1 protein did reveal more severe defects in axon guidance at the midline, the most well-established function of Netrin-1. In hypomorphic *Ntn1*$^{trap/trap}$ mice, guidance of commissural axons across the midline is severely disrupted and the ventral commissure is very thin (Serafini et al., 1996). For decades, it has been hypothesized that other axon guidance factors mediate the modest residual midline crossing observed in the absence of Netrin-1. Here, we show that in a true *Ntn1*$^{-/-}$ animal, almost no midline crossing of commissural axons is observed. This supports the idea that Netrin-1 is required for all midline axon guidance in the developing spinal cord, and raises the possibility that other phenotypes may also be more severe in *Ntn1*$^{-/-}$ animals.

One reason we created the null *Netrin-1* allele was to investigate whether loss of *Netrin-1* affects basement membrane integrity in tissues outside of the developing inner ear. In the future, the null allele can be used to examine basement membrane integrity in the complete absence of Netrin-1 protein during development of the lung, pancreas, and salivary gland, tissues where Netrins are present in the basement membrane and are involved in branching morphogenesis (Liu et al., 2004; Yebra et al., 2003; Schneiders et al., 2007). Netrin-1 is also involved in branching morphogenesis of the mammary gland

(Srinivasan et al., 2003), a tissue which develops postnatally. Tissue-specific Cre drivers can be used to remove Netrin-1 from mammary gland tissue determine if Netrin-1 acts to regulate basement membrane breakdown in this tissue.

The newly generated conditional floxed allele will also allow investigation of other postnatal roles for Netrin-1. Previous studies of Netrin-1 function after birth have relied either on transplantation techniques (Srinivasan et al., 2003) or *in vitro* culture of primary cells from mutant animals until they reach “maturity” (Goldman et al., 2013). While both techniques have enhanced our understanding of Netrin-1 function, studying complete protein loss in an intact *in vivo* environment will permit a more definitive assessment of Netrin-1 functions after birth.

Chapter 5: Concluding remarks and future directions

Netrins play many critical roles in development, with effects not only on axon attraction and repulsion, but also on neuronal survival and cell adhesion. Here, we sought to determine how Netrin-1 mediates its effects in one *in vivo* context: the morphogenesis of the semicircular canals of the inner ear. We provide evidence that Netrin-1 has divergent cellular effects during canal formation: in chicks it regulates cell survival while in the mouse it regulates basement membrane integrity. These findings highlight the complexity of Netrin-1 function *in vivo* and underscore the need to carefully delineate Netrin-1 function during development, adulthood, and disease.

Cellular events underlying complex tissue morphogenesis

Morphogenesis of a complex tissue requires the precise synchronization and coordination of many cellular processes in order to form the intricate structures that are necessary for life. A stunning example of this is the formation of the inner ear, which develops from a simple sphere of epithelium into an elaborate fluid-filled labyrinth featuring the semicircular canals of the vestibular system. Semicircular canal formation requires particularly careful coordination of cellular events. First, epithelial pockets called canal pouches grow out from the otic vesicle. Cells in the center of these pouches then form a specialized region called a fusion plate where the opposing epithelial layers intercalate into a single layer. These cells are eventually eliminated, leaving a donut shaped epithelial tube with mesenchymal cells in the center. The morphology of the developing inner ear is an exquisitely sensitive system for studying morphogenesis because the precise shape of the semicircular canals depends on the exact location and timing of fusion; thus, even subtle changes in fusion lead to easily observed morphological changes in canal structure

In chicks, widespread apoptosis occurs at the fusion plate and is required for canal formation (Fekete et al., 1997). Classically, apoptosis during tissue morphogenesis has been viewed as a way to remove unnecessary tissue, as occurs during elimination of interdigital webbing (Garcia-Martinez et al., 1993). Along these lines, apoptosis during chick canal development is hypothesized to remove fusion plate cells after fusion. The presence of apoptotic cells at the fusion plate well before fusion (Lang et al., 2000), however, suggests that cell death has functions besides fusion plate clearance. In most cases, blocking cell death by overexpressing Bcl2 delayed fusion plate clearance by up to 36 hours and resulted in wider diameter canals. In the most severe cases, overexpressing Bcl2 led to the formation of abnormally long fusion plates where the opposing epithelial layers had come into close contact but remained unfused, resulting in the retention of the canal pouch (Fekete et al., 1997). This suggests that apoptosis might play a role in the initiation of fusion. Along these lines, we showed that apoptosis precedes basement membrane breakdown during fusion, and that when apoptosis is inhibited by Netrin-1 overexpression, basement membrane breakdown and fusion fail to proceed. These results strongly suggest that in addition to clearing fusion plate cells after fusion, apoptosis is involved in early fusion plate formation. For example, aside from potentially initiating basement membrane breakdown, which has been hypothesized to allow for diffusion of secreted signaling molecules (Abraira et al., 2008), apoptosis at the fusion plate could also function early to disrupt epithelial cell-cell interactions and allow for the intercalation of the opposing epithelial layers.

Apoptosis may play an important role in the induction of the cellular reorganization which drives morphogenesis during vertebrate development, particularly

during tissue fusion events (reviewed in Ray and Niswander, 2012). Apoptosis at the fusion seam occurs during three well-studied fusion events: fusion of palatal shelves during secondary palate development (Mori et al., 1994; Cuervo et al., 2004), fusion of endocardial cushions during cardiac septation (Pexieder, 1975; Zhao and Rivkees, 2000), and neural tube closure (Geelen and Langman, 1979; Weil et al., 1997). As discussed in Chapter 2, apoptosis is required for fusion during palate formation (Cuervo et al., 2004). While the lack of *ex vivo* methods to study heart development has limited efforts to determine whether apoptosis is required for fusion during cardiac septation, recent advances have allowed for preliminary assessment of the role of apoptosis during neural tube closure.

During neural tube closure, elevated neural folds bend toward the midline and fuse together to form the roof of the neural tube. Extensive apoptosis occurs in the dorsal ridge of the neural plates and in the boundary between the neural plates and surface ectoderm (Weil et al., 1997; Yamaguchi et al., 2011). Neural tube closure initiates at many sites along the rostral-caudal axis and closure proceeds up and down from the initiation sites to “zip” the neural tube closed along its entire length. In chicks, blocking apoptosis prevents neural tube closure (Weil et al., 1997). On the other hand, blocking apoptosis in mice does not prevent neural tube closure entirely, but interferes with smooth zipping (Yamaguchi et al., 2011). While the underlying molecular machinery is unknown, it has been shown that apoptosis can directly influence the cytoskeleton of neighboring cells to form an actomyosin ring which squeezes the dying cell out of an epithelial layer (Rosenblatt et al., 2001). Modifications in the actomyosin network in a tissue could create a force to drive tissue movement (reviewed in Suzanne

and Steller 2013). As a whole, evidence from multiple systems suggests that apoptosis may play an important role in allowing tissue fusion, as we show in the developing chick inner ear.

A role for Netrin-1 as a trophic factor *in vivo*

Our work in the chick provides new evidence that Netrin-1 can act as a trophic factor *in vivo*, as overexpressing *Netrin-1* prevented the apoptosis that is required for fusion. Netrin-1 promotes survival *in vitro* by binding to DCC and Unc5, which trigger cell death in the absence of ligand (Mehlen et al., 1998). The ability of Netrin-1 to promote survival *in vivo*, however, is controversial. For example, some studies of Netrin-1 in the developing spinal cord reported increased cell death in *Netrin-1* mutants (Furne et al., 2008), while others report no change in cell death in *Netrin-1* mutants (Williams et al., 2006). In flies, NetB acts mainly as a trophic factor during midline guidance, while NetA acts a tropic guidance cue. Blocking apoptosis was able to rescue the midline guidance defects in *NetA/NetB* double mutants (Newquist et al., 2013), providing *in vivo* evidence that the survival and guidance activities of Netrin work together to eliminate inappropriately guided axons, thus ensuring the formation of appropriate neural connections (reviewed in Mehlen and Furne, 2005). Our work in the chick bolsters the evidence that Netrin-1 can promote survival *in vivo* during normal development and encourages investigation into Netrin-1 upregulation as a therapeutic target for cancer treatment. While final confirmation of the role of Netrin-1 as a trophic factor during chick canal morphogenesis will require loss of function studies, our gain of function studies are highly relevant to overexpression of *Netrin-1* in cancer.

Divergent roles for Netrin-1 during semicircular canal formation

Despite being highly conserved, Netrin proteins have a wide range of cellular effects *in vivo*. By signaling through multiple receptors, Netrins can influence guidance and migration, survival, and adhesion, which allows them to play important roles in many aspects of development. Further, Netrins can have different cellular mechanisms of action across species, even during the same developmental process. For example, during commissural midline crossing, Netrins act as short-range guidance cues in *Drosophila* (Brankatschk and Dickson, 2006) and long-range guidance cues in the mouse (Serafini et al., 1996). We suggest that Netrin-1 plays similarly divergent roles in canal formation between chicks and mice. The primary cellular effect of Netrin-1 during canal formation in chicks appear to be survival, while the primary cellular effect of Netrin-1 in mice appears to be regulation of basement membrane integrity. The role of Netrins in fusion in other species remains to be seen. In *Xenopus laevis* and *Danio rerio*, for example, epithelial protrusions grow out from the walls of the otic vesicle, meet, and fuse to form epithelial pillars, which separate the otic vesicle into the three semicircular canals (Waterman and Bell, 1984; Paterson1948). It will be interesting to examine the role of Netrins during fusion in these species.

Molecular mechanisms underlying Netrin-1 regulation of basement membrane integrity

The mechanism by which Netrin-1 mediates its effects on basement membrane integrity during canal formation in mice remains unknown. While we have clear evidence that Netrin-1 affects basement membrane integrity *in vivo* during semicircular

canal fusion in mice (Salminen et al., 2000; Abraira et al., 2008), no single Netrin-1 receptor seems to mediate Netrin-1's role in canal morphogenesis (Matilainen et al., 2007; Abraira et al., 2008). These negative results could be due to redundancy between Netrin receptors, the involvement of an unknown Netrin receptor, or could indicate that Netrin mediates its effects on canal formation directly in a receptor-independent manner. Structure-function studies on Netrin-1 itself may allow us to differentiate between these possibilities.

Specific functions have been attributed to specific domains of Netrin-1. The N-terminus, for example, binds Unc5 and DCC (Kruger et al., 2004; Xu et al., 2014), while the C-terminus interacts with extracellular matrix components such as heparin (Geisbrecht et al., 2003; Kappler et al., 2000), laminin (Schneiders et al., 2007), and type IV collagen (Yebra et al., 2003). Domains VI-V are sufficient for axon outgrowth from mammalian tissue explants *in vitro* (Mirzayan, 1998) and circumferential axon guidance in C. elegans *in vivo* (Wang and Wadsworth, 2002*)*. While the NTR domain may be disposable for axon guidance, selective loss of the NTR domain caused ectopic axon branching in C. elegans (Wang and Wadsworth, 2002), demonstrating a role for the NTR domain *in vivo*. We hypothesize that the NTR domain of Netrin-1 mediates its effects on basement membrane integrity during canal morphogenesis.

The NTR domain shares homology with tissue inhibitors of metalloproteinases (TIMPs), complement C3, 4, and 5, secreted frizzled related proteins (sFRPs), and type I C-proteinase enhancer proteins (PCOLCEs) (Banyai and Patthy, 1999). NTR domain containing proteins interact with various matrix metalloproteinases, the enzymes responsible for basement membrane breakdown. For example, sFRPs, which were

initially characterized as modulators of Wnt signaling, have been shown to regulate matrix metalloproteinases. In mammals, sFRPs enhance the procollagen C-proteinase activity of tolloid-like matrix metalloproteinases (Kobayashi et al., 2009). In *Xenopus* and fish, on the other hand, sFRPs inhibit tolloid-like matrix metalloproteinases (Lee at al., 2006; Muraoka et al., 2006). Other examples of NTR domains inhibiting matrix metalloproteinase activity include the NTR domain of PCOLCEs, which is released through proteolytic processing and acts as a metalloproteinase inhibitor (Mott et al., 2000), and TIMPs, where the NTR domain directly binds and inhibits matrix metalloproteinases (Murphy et al., 1995). These examples suggest that the NTR domain of Netrin-1 may also modulate matrix metalloproteinases to regulate basement membrane integrity.

Another possibility is that the NTR domain is responsible for changes in cell morphology via integrin signaling, as the C-terminus of Netrin-1 binds integrins to mediate epithelial adhesion in the developing pancreas (Yebra et al., 2003). The involvement of integrins may be less likely since previous work has shown normal canal morphology in mutants for both integrins α6 and α3 (Abraria et al., 2008), although analysis on double mutants has not been performed and integrin α6 and α3 have been shown to act redundantly in other systems (De Arcangelis et al., 1999).

To test if a single domain of Netrin-1 is sufficient to regulate basement membrane breakdown during canal morphogenesis, both gain of function and loss of function approaches can be taken. With the advent of CRISPR/Cas-9 mediated genome engineering (Wang et al., 2013), creating mouse models for structure-function studies is much faster. We have already begun structure-function studies in the chick to determine

if overexpression of either the N-terminus or C-terminus of Netrin-1 is sufficient to replicate the effects of overexpression of full-length Netrin-1 on semicircular canal morphogenesis. Recent reports demonstrate the existence of both N-terminal and C-terminal fragments of Netrin-1 (Bin et al., 2013; Delloye-Bourgeois et al., 2012), highlighting the importance of *in vivo* Netrin-1 structure-function studies.

Creation of genetic tools to study Netrin-1 function in development and disease

The creation of the *Ntn1*^{CE} and *floxed Ntn1* alleles represents an important step towards understanding the complex functions of Netrin-1 *in vivo*. Previous experiments have used hypomorphic alleles of *Netrin-1* with residual amount of Netrin-1 protein. By generating a true null mutant we can better reveal the true roles of Netrin-1 in development. In *Netrin-1* hypomorphs, a small number of commissural axons project normally and cross the midline (Serafini et al., 1996). In our newly generated null, however, complete loss of Netrin-1 protein prevents all commissural axon crossing. Thus, Netrin-1 appears to be required for all commissural axon guidance to the midline, a function that was masked by the small amount of Netrin-1 protein in *Netrin-1* hypomorphs.

Similarly, while previous reports showed that spiral ganglion neurons can grow towards to Netrin-1 *in vitro* (Lee and Warchol, 2007), the spiral ganglion appears normal in *Netrin-1* hypomorphs (Salminen et al, 2000). In contrast, preliminary analysis of our newly generated *Ntn1*$^{-/-}$ mice shows ectopic neurons in the spiral ganglion (A. Yung, personal communication). These ectopic neurons are positive for Gata3, an early spiral ganglion neuron marker, but not MafB, a mature spiral ganglion marker. To better

understand the role of Netrin-1 in the cochlea, future research will carefully characterize the spatiotemporal expression patterns of *Netrin-1* and its receptors during inner ear development. Continuing work will also determine the effect of these ectopic neurons on the highly stereotyped wiring of the cochlea and determine whether they are generated by changes in cell proliferation, cell death, or migration.

The newly generated conditional floxed allele will also allow for the investigation of postnatal roles for Netrin-1. *Netrin-1* expression is maintained in the adult nervous system, as well as several other tissues (reviewed in Manitt and Kennedy, 2002; Kennedy et al., 1994). In the adult spinal cord, *Netrin-1* is expression is maintained at a level similar to that in embryonic development (Manitt et al, 2001). At this stage, the majority of Netrin-1 protein is associated with cell membranes or the extracellular matrix, suggesting that it mediates cell-cell interactions (Manitt et al., 2001), in particular between neurons and myelinating glia: both peripheral Schwann cells (Madison et al., 2000; Ellezam et al., 2001) and central oligodendroctyes (Manitt et al., 2001) express *Netrin-1*.

Netrin-1 expression in the adult nervous system also has important implications for injury and disease. For example, expression of *Netrin-1* by oligodendrocytes may act as a myelin-associated inhibitor of axonal regeneration (reviewed in Mannit and Kennedy, 2002). Intriguingly, expression of the attractive Netrin-1 receptor DCC is lower while expression of the repulsive Netrin-1 receptor Unc5 is higher in the adult when compared to expression levels in the embryonic spinal cord (Manitt and Kennedy, 2002). This suggests that in the adult Netrin-1 may be primarily a repulsive axon guidance cue. In support of this, axonal regeneration in the lamprey spinal cord is

inversely correlated with expression of Unc5 in regenerating axons (Shifman and Selzer, 2000).

Netrin-1 may also be a therapeutic target for multiple sclerosis, an autoimmune disease where myelination is degraded. *Netrin-1* is expressed in normal human brain white matter as well as in multiple sclerosis lesions (Bin et al., 2013). During development, Netrin-1 repels oligodendrocyte precursor cells (Jarjour et al., 2003; Tsai et al., 2003) and *in vitro* migration assays showed that adult oligodendrocyte precursor cells, which express Netrin-1 receptors, respond similarly (Tepavčević et al., 2014). In human multiple sclerosis lesions, astrocytes upregulate Netrin-1 during demyelination. This corresponds to mouse models of multiple sclerosis, where overexpression of *Netrin-1* reduced oligodendrocyte precursor cell recruitment and remyelination. Conversely, antibody-mediated disruption of Netrin-1 function increased oligodendrocyte precursor cell recruitment to the lesion, thus promoting remyelination (Tepavčević et al., 2014). These data identify Netrin-1 as a candidate inhibitor of remyelination during multiple sclerosis.

Netrin-1 has multiple critical functions during development and disease. Fully understanding its complex roles *in vivo* have been limited by the lack of genetic tools to increase and decrease Netrin-1 levels in a tissue specific manner. The combination of the conditional expression allele and conditional null allele of *Netrin-1* presented in this work will serve as an important tool to further our understanding of Netrin-1 function from development to adulthood.

References

Abraira VE, Del Rio T, Tucker AF, Slonimsky J, Keirnes HL, Goodrich LV. (2008). *Cross-repressive interactions between Lrig3 and netrin 1 shape the architecture of the inner ear.* Development. 135(24):4091-9.

Abraira VE, Satoh T, Fekete DM, Goodrich LV. (2010). *Vertebrate Lrig3-ErbB interactions occur in vitro but are unlikely to play a role in Lrig3-dependent inner ear morphogenesis.* PLoS One. 5(2):e8981

Andrews GL, Tanglao S, Farmer WT, Morin S, Brotman S, Berberoglu MA, Price H, Fernandez GC, Mastick GS, Charron F, Kidd T. (2008). *Dscam guides embryonic axons by Netrin-dependent and -independent functions.* Development. 135(23):3839-48.

Anniko M, Wikström SO. (1984). *Pattern formation of the otic placode and morphogenesis of the otocyst.* Am J Otolaryngol. 5(6):373-81.

Baker KA, Moore SW, Jarjour AA, Kennedy TE. (2006). *When a diffusible axon guidance cue stops diffusing: roles for netrins in adhesion and morphogenesis.* Curr Opin Neurobiol.16(5):529-34.

Bányai L, Patthy L. (1999). *The NTR module: domains of netrins, secreted frizzled related proteins, and type I procollagen C-proteinase enhancer protein are homologous with tissue inhibitors of metalloproteases.* Protein Sci. 8(8):1636-42.

Bin JM, Rajasekharan S, Kuhlmann T, Hanes I, Marcal N, Han D, Rodrigues SP, Leong SY, Newcombe J, Antel JP, Kennedy TE. (2013). *Full-length and fragmented netrin-1 in multiple sclerosis plaques are inhibitors of oligodendrocyte precursor cell migration.* Am J Pathol.183(3):673-80.

Bok J, Chang W, Wu DK. (2007). *Patterning and morphogenesis of the vertebrate inner ear.* Int J Dev Biol. 51(6-7):521-33.

Brankatschk M, Dickson BJ. (2006). *Netrins guide Drosophila commissural axons at short range.* Nat Neurosci. 9(2):188-94.

Burgess RW, Jucius TJ, Ackerman SL. (2006). *Motor axon guidance of the mammalian trochlear and phrenic nerves: dependence on the netrin receptor Unc5c and modifier loci.* J Neurosci. 26(21):5756-66.

Chan SS, Zheng H, Su MW, Wilk R, Killeen MT, Hedgecock EM, Culotti JG. (1996). *UNC-40, a C. elegans homolog of DCC (Deleted in Colorectal Cancer), is required in motile cells responding to UNC-6 netrin cues.* Cell. 87(2):187-95.

Chang W, Brigande JV, Fekete DM, Wu DK. (2004). *The development of semicircular canals in the inner ear: role of FGFs in sensory cristae.* Development. 131(17):4201-11.

Chiarugi P, Giannoni E. (2008). *Anoikis: a necessary death program for anchorage-dependent cells.* Biochem Pharmacol. 76(11):1352-64.

Colamarino SA, Tessier-Lavigne M. (1995). *The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons.* Cell. 81(4):621-9.

Cuervo R, Valencia C, Chandraratna RA, Covarrubias L. (2002). *Programmed cell death is required for palate shelf fusion and is regulated by retinoic acid.* Dev Biol. 245(1):145-56.

Cuervo R, Covarrubias L. (2004). *Death is the major fate of medial edge epithelial cells and the cause of basal lamina degradation during palatogenesis.* Development. 131(1):15-24.

De Arcangelis A, Mark M, Kreidberg J, Sorokin L, Georges-Labouesse E. (1999). *Synergistic activities of alpha3 and alpha6 integrins are required during apical ectodermal ridge formation and organogenesis in the mouse.* Development. 126(17):3957-68.

de la Torre JR, Höpker VH, Ming GL, Poo MM, Tessier-Lavigne M, Hemmati-Brivanlou A, Holt CE. (1997). *Turning of retinal growth cones in a netrin-1 gradient mediated by the netrin receptor DCC.* Neuron. 19(6):1211-24.

del Rio T, Nishitani AM, Yu WM, Goodrich LV. (2013). *In vivo analysis of Lrig genes reveals redundant and independent functions in the inner ear.* PLoS Genet. 9(9):e1003824.

Delloye-Bourgeois C, Fitamant J, Paradisi A, Cappellen D, Douc-Rasy S, Raquin MA, Stupack D, Nakagawara A, Rousseau R, Combaret V, Puisieux A, Valteau-Couanet D, Bénard J, Bernet A, Mehlen P. (2009). *Netrin-1 acts as a survival factor for aggressive neuroblastoma.* J Exp Med. 206(4):833-47.

Delloye-Bourgeois C, Brambilla E, Coissieux MM, Guenebeaud C, Pedeux R, Firlej V, Cabon F, Brambilla C, Mehlen P, Bernet A. (2009). *Interference with netrin-1 and tumor cell death in non-small cell lung cancer.* J Natl Cancer Inst. 101(4):237-47.

Delloye-Bourgeois C, Goldschneider D, Paradisi A, Therizols G, Belin S, Hacot S, Rosa-Calatrava M, Scoazec JY, Diaz JJ, Bernet A, Mehlen P. (2012). *Nucleolar localization of a netrin-1 isoform enhances tumor cell proliferation.* Sci Signal. 5(236):ra57.

Ellezam B, Selles-Navarro I, Manitt C, Kennedy TE, McKerracher L. (2001). *Expression of netrin-1 and its receptors DCC and UNC-5H2 after axotomy and during regeneration of adult rat retinal ganglion cells.* Exp Neurol.168(1):105-15.

Fazeli A, Dickinson SL, Hermiston ML, Tighe RV, Steen RG, Small CG, Stoeckli ET, Keino-Masu K, Masu M, Rayburn H, Simons J, Bronson RT, Gordon JI, Tessier-Lavigne M, Weinberg RA. (1997). *Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene.* Nature. 386(6627):796-804.

Fearon ER, Cho KR, Nigro JM, Kern SE, Simons JW, Ruppert JM, Hamilton SR, Preisinger AC, Thomas G, Kinzler KW, et al. (1990). *Identification of a chromosome 18q gene that is altered in colorectal cancers.* Science. 247(4938):49-56.

Fekete DM, Homburger SA, Waring MT, Riedl AE, Garcia LF. (1997). *Involvement of programmed cell death in morphogenesis of the vertebrate inner ear.* Development. 124(12):2451-61.

Fitamant J, Guenebeaud C, Coissieux MM, Guix C, Treilleux I, Scoazec JY, Bachelot T, Bernet A, Mehlen P. (2008). *Netrin-1 expression confers a selective advantage for tumor cell survival in metastatic breast* Proc Natl Acad Sci USA. 105(12):4850-5.

Fitzky BU, Witsch-Baumgartner M, Erdel M, Lee JN, Paik YK, Glossmann H, Utermann G, Moebius FF. (1998). *Mutations in the Delta7-sterol reductase gene in patients with the Smith-Lemli-Opitz syndrome.* Proc Natl Acad Sci USA. 7;95(14):8181-6.

Forcet C, Ye X, Granger L, Corset V, Shin H, Bredesen DE, Mehlen P. (2001). *The dependence receptor DCC (deleted in colorectal cancer) defines an alternative mechanism for caspase activation.* Proc Natl Acad Sci USA. 98(6):3416-21.

Forni PE, Taylor-Burds C, Melvin VS, Williams T, Wray S. (2011). *Neural crest and ectodermal cells intermix in the nasal placode to give rise to GnRH-1 neurons, sensory neurons, and olfactory ensheathing cells.* J Neurosci. 31(18):6915-27.

Fuchs Y, Steller H. (2011). *Programmed cell death in animal development and disease.* Cell. 147(4):742-58.

Garcia-Martinez V, Macias D, Gañan Y, Garcia-Lobo JM, Francia MV, Fernandez-Teran MA, Hurle JM. (1993). *Internucleosomal DNA fragmentation and programmed cell death (apoptosis) in the interdigital tissue of the embryonic chick leg bud.* J Cell Sci. 106:201-8.

Geelen JA, Langman J. (1979). *Ultrastructural observations on closure of the neural tube in the mouse.* Anat Embryol (Berl). 156(1):73-88.

Geisbrecht BV, Dowd KA, Barfield RW, Longo PA, Leahy DJ. (2003). *Netrin binds discrete subdomains of DCC and UNC5 and mediates interactions between DCC and heparin.* J Biol Chem. 278(35):32561-8.

Grossmann J. (2002). *Molecular mechanisms of "detachment-induced apoptosis--Anoikis".* Apoptosis. 7(3):247-60.

Groves AK, Fekete DM. (2012). *Shaping sound in space: the regulation of inner ear patterning.* Development. 139(2):245-57.

Goldman JS, Ashour MA, Magdesian MH, Tritsch NX, Harris SN, Christofi N, Chemali R, Stern YE, Thompson-Steckel G, Gris P, Glasgow SD, Grutter P, Bouchard JF, Ruthazer ES, Stellwagen D, Kennedy TE. (2013). *Netrin-1 promotes excitatory synaptogenesis between cortical neurons by initiating synapse assembly.* J Neurosci. 33(44):17278-89

Haddon CM, Lewis JH. (1991). *Hyaluronan as a propellant for epithelial movement: the development of semicircular canals in the inner ear of Xenopus.* Development. 112(2):541-50.

Hagedorn EJ, Ziel JW, Morrissey MA, Linden LM, Wang Z, Chi Q, Johnson SA, Sherwood DR. (2013). *The netrin receptor DCC focuses invadopodia-driven basement membrane transmigration in vivo.* J Cell Biol. 201(6):903-13.

Harlow DE, Yang H, Williams T, Barlow LA. (2011). *Epibranchial placode-derived neurons produce BDNF required for early sensory neuron development.* Dev Dyn. 240(2):309-23.

Harris R, Sabatelli LM, Seeger MA. (1996). *Guidance cues at the Drosophila CNS midline: identification and characterization of two Drosophila Netrin/UNC-6 homologs.* Neuron. 17(2):217-28.

Hébert JM, McConnell SK. (2000). *Targeting of cre to the Foxg1 (BF-1) locus mediates loxP recombination in the telencephalon and other developing head structures.* Dev Biol. 222(2):296-306.

Hedgecock EM, Culotti JG, Hall DH. (1990). *The unc-5, unc-6, and unc-40 genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in C. elegans.* Neuron. 4(1):61-85.

Hollenbeck PJ, Fekete DM. (2003). *Expression of transgenes in primary neurons from chick peripheral and central nervous systems by retroviral infection of early embryos.* Methods Cell Biol. 71:369-86.

Hong K, Hinck L, Nishiyama M, Poo MM, Tessier-Lavigne M, Stein E. (1999). *A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion.* Cell. 97(7):927-41.

Ishii N, Wadsworth WG, Stern BD, Culotti JG, Hedgecock EM. (1992). *UNC-6, a laminin-related protein, guides cell and pioneer axon migrations in C. elegans.* Neuron. 9(5):873-81.

Jarjour AA, Manitt C, Moore SW, Thompson KM, Yuh SJ, Kennedy TE. (2003). *Netrin-1 is a chemorepellent for oligodendrocyte precursor cells in the embryonic spinal cord.* J Neurosci. 23(9):3735-44.

Kappler J, Franken S, Junghans U, Hoffmann R, Linke T, Müller HW, Koch KW. (2000).*Glycosaminoglycan-binding properties and secondary structure of the C-terminus of netrin-1.* Biochem Biophys Res Commun. 271(2):287-91.

Keino-Masu K, Masu M, Hinck L, Leonardo ED, Chan SS, Culotti JG, Tessier-Lavigne M. (1996). *Deleted in Colorectal Cancer (DCC) encodes a netrin receptor.* Cell. 87(2):175-85.

Keleman K, Dickson BJ. (2001). *Short- and long-range repulsion by the Drosophila Unc5 netrin receptor.* Neuron. 32(4):605-17.

Kennedy TE, Serafini T, de la Torre JR, Tessier-Lavigne M. (1994). *Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord.* Cell. 78(3):425-35.

Kennedy TE, Wang H, Marshall W, Tessier-Lavigne M. (2006). *Axon guidance by diffusible chemoattractants: a gradient of netrin protein in the developing spinal cord.* J Neurosci. 26(34):8866-74.

Kim SK, Hebrok M. (2001). *Intercellular signals regulating pancreas development and function.* Genes Dev. 15(2):111-27.

Kobayashi Y, Nakamura H, Funahashi J. (2008). *Epithelial-mesenchymal transition as a possible mechanism of semicircular canal morphogenesis in chick inner ear.* Tohoku J Exp Med. 215(3):207-17.

Kobayashi K, Luo M, Zhang Y, Wilkes DC, Ge G, Grieskamp T, Yamada C, Liu TC, Huang G, Basson CT, Kispert A, Greenspan DS, Sato TN. (2008). *Secreted Frizzled-related protein 2 is a procollagen C proteinase enhancer with a role in fibrosis associated with myocardial infarction.* Nat Cell Biol.11(1):46-55.

Kruger RP, Lee J, Li W, Guan KL. (2004). *Mapping netrin receptor binding reveals domains of Unc5 regulating its tyrosine phosphorylation.* J Neurosci. 24(48):10826-34.

Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, Lee E, Alt FW, Westphal H. (1996). *Efficient in vivo manipulation of mouse genomic sequences at the zygote stage.* Proc Natl Acad Sci USA. 93(12):5860-5.

Lang H, Bever MM, Fekete DM. (2002). *Cell proliferation and cell death in the developing chick inner ear: spatial and temporal patterns.* J Comp Neurol. 417(2):205-20.

Lauderdale JD, Davis NM, Kuwada JY. (1997). *Axon tracts correlate with netrin-1a expression in the zebrafish embryo.* Mol Cell Neurosci. 9(4):293-313.

Lee KH, Warchol ME. (2008). *Promotion of neurite outgrowth and axon guidance in spiral ganglion cells by netrin-1.* Arch Otolaryngol Head Neck Surg.134(2):146-51.

Lee, H. X., Ambrosio, A. L., Reversade, B. & De Robertis, E. M. (2006). *Embryonic dorsal-ventral signaling: secreted frizzled-related proteins as inhibitors of tolloid proteinases.* Cell. 124:147–159

Leonardo ED, Hinck L, Masu M, Keino-Masu K, Ackerman SL, Tessier-Lavigne M. (1997). *Vertebrate homologues of C. elegans UNC-5 are candidate netrin receptors.* Nature. 386(6627):833-8.

Leung-Hagesteijn C, Spence AM, Stern BD, Zhou Y, Su MW, Hedgecock EM, Culotti JG. (1992). *UNC-5, a transmembrane protein with immunoglobulin and thrombospondin type 1 domains, guides cell and pioneer axon migrations in C. elegans.* Cell. 71(2):289-99.

Link BC, Reichelt U, Schreiber M, Kaifi JT, Wachowiak R, Bogoevski D, Bubenheim M, Cataldegirmen G, Gawad KA, Issa R, Koops S, Izbicki JR, Yekebas EF. (2007). *Prognostic implications of netrin-1 expression and its receptors in patients with adenocarcinoma of the pancreas.* Ann Surg Oncol. 14(9):2591-9.

Liu Y, Stein E, Oliver T, Li Y, Brunken WJ, Koch M, Tessier-Lavigne M, Hogan BL. (2004). *Novel role for Netrins in regulating epithelial behavior during lung branching morphogenesis.* Curr Biol. 14(10):897-905.

Llambi F, Causeret F, Bloch-Gallego E, Mehlen P. (2001). *Netrin-1 acts as a survival factor via its receptors UNC5H and DCC.* EMBO J. 20(11):2715-22

Lu X, Le Noble F, Yuan L, Jiang Q, De Lafarge B, Sugiyama D, Bréant C, Claes F, De Smet F, Thomas JL, Autiero M, Carmeliet P, Tessier-Lavigne M, Eichmann A. (2004). *The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system.* Nature. 432(7014):179-86.

Ly A, Nikolaev A, Suresh G, Zheng Y, Tessier-Lavigne M, Stein E. (2008). *DSCAM is a netrin receptor that collaborates with DCC in mediating turning responses to netrin-1.* Cell. 133(7):1241-54.

Madison RD, Zomorodi A, Robinson GA. (2000). *Netrin-1 and peripheral nerve regeneration in the adult rat.* Exp Neurol.161(2):563-70.

Manitt C, Colicos MA, Thompson KM, Rousselle E, Peterson AC, Kennedy TE. (2001). *Widespread expression of netrin-1 by neurons and oligodendrocytes in the adult mammalian spinal cord.* J Neurosci. 21(11):3911-22.

Manitt C, Kennedy TE. (2002). *Where the rubber meets the road: netrin expression and function in developing and adult nervous systems.* Prog Brain Res. 137:425-42.

Martin P, Swanson GJ. (1993). *Descriptive and experimental analysis of the epithelial remodellings that control semicircular canal formation in the developing mouse inner ear.* Dev Biol. 159(2):549-58.

Matilainen T, Haugas M, Kreidberg JA, Salminen M. (2007). *Analysis of Netrin 1 receptors during inner ear development.* Int J Dev Biol. 51(5):409-13.

Matus DQ, Pang K, Marlow H, Dunn CW, Thomsen GH, Martindale MQ. (2006). *Molecular evidence for deep evolutionary roots of bilaterality in animal development.* Proc Natl Acad Sci USA. 103(30):11195-200.

Mazelin L, Bernet A, Bonod-Bidaud C, Pays L, Arnaud S, Gespach C, Bredesen DE, Scoazec JY, Mehlen P. (2004). *Netrin-1 controls colorectal tumorigenesis by regulating apoptosis.* Nature. 431(7004):80-4.

Mehlen P, Rabizadeh S, Snipas SJ, Assa-Munt N, Salvesen GS, Bredesen DE. (1998). *The DCC gene product induces apoptosis by a mechanism requiring receptor proteolysis.* Nature. 395(6704):801-4.

Mehlen P, Furne C. (2005). *Netrin-1: when a neuronal guidance cue turns out to be a regulator of tumorigenesis.* Cell Mol Life Sci. 62(22):2599-616.

Mehlen P, Delloye-Bourgeois C, Chédotal A. (2011). *Novel roles for Slits and netrins: axon guidance cues as anticancer targets?* Nat Rev Cancer. 11(3):188-97.

Métin C, Deléglise D, Serafini T, Kennedy TE, Tessier-Lavigne M. (1997). *A role for netrin-1 in the guidance of cortical efferents.* Development.124(24):5063-74.

Meyerhardt JA, Caca K, Eckstrand BC, Hu G, Lengauer C, Banavali S, Look AT, Fearon ER. (1999). *Netrin-1: interaction with deleted in colorectal cancer (DCC) and alterations in brain tumors and neuroblastomas.* Cell Growth Differ. 10(1):35-42.

Mirzayan, C. (1998). *Molecular mechanisms underlying the response of commissural axons to Netrin-1.* Doctoral dissertation, University of California, San Francisco.

Mitchell KJ, Doyle JL, Serafini T, Kennedy TE, Tessier-Lavigne M, Goodman CS, Dickson BJ. (1996). *Genetic analysis of Netrin genes in Drosophila: Netrins guide CNS commissural axons and peripheral motor axons.* Neuron. 17(2):203-15.

Moore SW, Biais N, Sheetz MP. (2009). *Traction on immobilized netrin-1 is sufficient to reorient axons.* Science. 325(5937):166.

Mori C, Nakamura N, Okamoto Y, Osawa M, Shiota K. (1994). *Cytochemical identification of programmed cell death in the fusing fetal mouse palate by specific labelling of DNA fragmentation.* Anat Embryol (Berl). 190(1):21-8.

Mott JD, Thomas CL, Rosenbach MT, Takahara K, Greenspan DS, Banda MJ. (2000).*Post-translational proteolytic processing of procollagen C-terminal proteinase enhancer releases a metalloproteinase inhibitor.* J Biol Chem. 275(2):1384-90.

Muraoka O, Shimizu T, Yabe T, Nojima H, Bae YK, Hashimoto H, Hibi M. (2006). *Sizzled controls dorso-ventral polarity by repressing cleavage of the Chordin protein.* Nat Cell Biol. 8(4):329-38.

Murphy G, Houbrechts A, Cockett MI, Williamson RA, O'Shea M, Docherty AJ. (1991). *The N-terminal domain of tissue inhibitor of metalloproteinases retains metalloproteinase inhibitory activity.* Biochemistry. 30(33):8097-102.

Nakashiba T, Ikeda T, Nishimura S, Tashiro K, Honjo T, Culotti JG, Itohara S. (2000). *Netrin-G1: a novel glycosyl phosphatidylinositol-linked mammalian netrin that is functionally divergent from classical netrins.* J Neurosci. 20(17):6540-50.

Newquist G, Drennan JM, Lamanuzzi M, Walker K, Clemens JC, Kidd T. (2013). *Blocking apoptotic signaling rescues axon guidance in Netrin mutants.* Cell Rep. 3(3):595-606.

Nishikori T, Hatta T, Kawauchi H, Otani H. (1999). *Apoptosis during inner ear development in human and mouse embryos: an analysis by computer-assisted three-dimensional reconstruction.* Anat Embryol (Berl). 200(1):19-26.

Palmesino E, Haddick PC, Tessier-Lavigne M, Kania A. (2012). *Genetic analysis of DSCAM's role as a Netrin-1 receptor in vertebrates.* J Neurosci. 32(2):411-6.

Park KW, Crouse D, Lee M, Karnik SK, Sorensen LK, Murphy KJ, Kuo CJ, Li DY. (2004). *The axonal attractant Netrin-1 is an angiogenic factor.* Proc Natl Acad Sci USA. 101(46):16210-5.

Park KW, Urness LD, Senchuk MM, Colvin CJ, Wythe JD, Chien CB, Li DY. (2005). *Identification of new netrin family members in zebrafish: developmental expression of netrin 2 and netrin 4.* Dev Dyn. 234(3):726-31.

Paterson, NF. (1948). *The development of the inner ear or xenopus laevis*. Proc. Zool. Soc. Lond. 119:269-291.

Pauley S, Wright TJ, Pirvola U, Ornitz D, Beisel K, Fritzsch B. (2003). *Expression and function of FGF10 in mammalian inner ear development.* Dev Dyn. 227(2):203-15.

Pirvola U, Zhang X, Mantela J, Ornitz DM, Ylikoski J. (2004). *Fgf9 signaling regulates inner ear morphogenesis through epithelial-mesenchymal interactions.* Dev Biol. 273(2):350-60.

Puelles E, Acampora D, Lacroix E, Signore M, Annino A, Tuorto F, Filosa S, Corte G, Wurst W, Ang SL, Simeone A. (2003). *Otx dose-dependent integrated control of antero-posterior and dorso-ventral patterning of midbrain*. Nat Neurosci 6(5):453-60.

Ray HJ, Niswander L. (2012). *Mechanisms of tissue fusion during development.* Development. 139(10):1701-11

Rodríguez C, Buchholz F, Galloway J, Sequerra R, Kasper J, Ayala R, Stewart AF, Dymecki SM. (2000). *High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP.* Nat Genet. 25(2):139-40.

Rosenblatt J, Raff MC, Cramer LP. (2001). *An epithelial cell destined for apoptosis signals its neighbors to extrude it by an actin- and myosin-dependent mechanism.* Curr Biol. 11(23):1847-57.

Salminen M, Meyer BI, Bober E, Gruss P. (2000). *Netrin 1 is required for semicircular canal formation in the mouse inner ear.* Development. 127(1):13-22.

Sando I, Orita Y, Miura M, Balaban CD. (2001). *Vestibular abnormalities in congenital disorders.* Ann N Y Acad Sci. 942:15-24.

Schneiders FI, Maertens B, Böse K, Li Y, Brunken WJ, Paulsson M, Smyth N, Koch M. (2007). *Binding of netrin-4 to laminin short arms regulates basement membrane assembly.* J Biol Chem. 282(33):23750-8.

Serafini T, Kennedy TE, Galko MJ, Mirzayan C, Jessell TM, Tessier-Lavigne M. (1994). *The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6.* Cell. 78(3):409-24.

Serafini T, Colamarino SA, Leonardo ED, Wang H, Beddington R, Skarnes WC, Tessier-Lavigne M. (1996). *Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system.* Cell. 87(6):1001-14.

Setó-Salvia N, Stanier P. (2014). *Genetics of cleft lip and/or cleft palate: Association with other common anomalies.* Eur J Med Genet. S1769-7212(14)00088-3.

Shifman MI, Selzer ME. (2000). *Expression of the netrin receptor UNC-5 in lamprey brain: modulation by spinal cord transection.* Neurorehabil Neural Repair.14(1):49-58.

Skarnes WC, Moss JE, Hurtley SM, Beddington RS. (1995). *Capturing genes encoding membrane and secreted proteins important for mouse development.* Proc Natl Acad Sci USA. 92(14):6592-6.

Smyth N, Vatansever HS, Murray P, Meyer M, Frie C, Paulsson M, Edgar D. (1999). *Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation.* J Cell Biol. 144(1):151-60.

Srinivasan K, Strickland P, Valdes A, Shin GC, Hinck L. (2003). *Netrin-1/neogenin interaction stabilizes multipotent progenitor cap cells during mammary gland morphogenesis.* Dev Cell. 4(3):371-82.

Stein E, Zou Y, Poo M, Tessier-Lavigne M. (2001). *Binding of DCC by netrin-1 to mediate axon guidance independent of adenosine A2B receptor activation.* Science. 291(5510):1976-82.

Strähle U, Fischer N, Blader P. (1997). *Expression and regulation of a netrin homologue in the zebrafish embryo.* Mech Dev. 62(2):147-60.

Suzanne M, Steller H. (2013). *Shaping organisms with apoptosis.* Cell Death Differ. 20(5):669-75.

Tepavčević V, Kerninon C, Aigrot MS, Meppiel E, Mozafari S, Arnould-Laurent R, Ravassard P, Kennedy TE, Nait-Oumesmar B, Lubetzki C. (2014). *Early netrin-1 expression impairs central nervous system remyelination.* Ann Neurol. [Epub ahead of print].

Tsai HH, Tessier-Lavigne M, Miller RH. (2003). *Netrin 1 mediates spinal cord oligodendrocyte precursor dispersal.* Development. 2003. 130(10):2095-105.

Vielmetter J, Kayyem JF, Roman JM, Dreyer WJ. (1994). *Neogenin, an avian cell surface protein expressed during terminal neuronal differentiation, is closely related to the human tumor suppressor molecule deleted in colorectal cancer.* J Cell Biol. 127:2009-20.

Wang Q, Wadsworth WG (2002). *The C domain of netrin UNC-6 silences calcium/calmodulin-dependent protein kinase- and diacylglycerol-dependent axon branching in Caenorhabditis elegans.* J. Neurosci. 22(6):2274-82.

Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R. (2013). *One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering.* Cell. 153(4):910-8.

Waterman RE, Bell DH. (1984). *Epithelial fusion during early semicircular canal formation in the embryonic zebrafish, Brachydanio rerio.* Anat Rec. 210(1):101-14.

Weil M, Jacobson MD, Raff MC. (1997). *Is programmed cell death required for neural tube closure?* Curr Biol. 7(4):281-4.

Williams ME, Lu X, McKenna WL, Washington R, Boyette A, Strickland P, Dillon A, Kaprielian Z, Tessier-Lavigne M, Hinck L. (2006). *UNC5A promotes neuronal apoptosis during spinal cord development independent of netrin-1.* Nat Neurosci. 9(8):996-8.

Wu DK, Kelley MW. (2012). *Molecular mechanisms of inner ear development.* Cold Spring Harb Perspect Biol. 4(8):a008409.

Xu K, Wu Z, Renier N, Antipenko A, Tzvetkova-Robev D, Xu Y, Minchenko M, Nardi-Dei V, Rajashankar KR, Himanen J, Tessier-Lavigne M, Nikolov DB. (2014). *Structures of netrin-1 bound to two receptors provide insight into its axon guidance mechanism.* Science. 344(6189):1275-9.

Yamaguchi Y, Shinotsuka N, Nonomura K, Takemoto K, Kuida K, Yosida H, Miura M. (2011). *Live imaging of apoptosis in a novel transgenic mouse highlights its role in neural tube closure.* J Cell Biol. 195(6):1047-60.

Yebra M, Montgomery AM, Diaferia GR, Kaido T, Silletti S, Perez B, Just ML, Hildbrand S, Hurford R, Florkiewicz E, Tessier-Lavigne M, Cirulli V. (2003). *Recognition of the neural chemoattractant Netrin-1 by integrins alpha6beta4 and alpha3beta1 regulates epithelial cell adhesion and migration.* Dev Cell, 5(5):695-707.

Yu WM, Appler JM, Kim YH, Nishitani AM, Holt JR, Goodrich LV. (2013). *A Gata3-Mafb transcriptional network directs post-synaptic differentiation in synapses specialized for hearing.* Elife. 10;2:e01341.

Yurchenco PD, Wadsworth WG. (2004). *Assembly and tissue functions of early embryonic laminins and netrins.* Curr Opin Cell Biol. 16(5):572-9.

Zhao Z, Rivkees SA. (2000). *Programmed cell death in the developing heart: regulation by BMP4 and FGF2.* Dev Dyn. 217(4):388-400.

Zhao H, Tanegashima K, Ro H, Dawid IB. (2008). *Lrig3 regulates neural crest formation in Xenopus by modulating Fgf and Wnt signaling pathways.* Development. 135(7):1283-93.

Ziel JW, Hagedorn EJ, Audhya A, Sherwood DR. (2009). *UNC-6 (netrin) orients the invasive membrane of the anchor cell in C. elegans.* Nat Cell Biol. 11(2):183-9.